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STUDIES ON THE LARVAL SALIVARY GLAND OF *DROSOPHILA*

I. THE NUCLEIC ACIDS

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Received September 6, 1950

The present study deals with the nucleic acids of the salivary gland of *Drosophila robusta* during the course of its larval development. Growth of most dipteran larval organs is through an increase in cell size rather than an increase in cell number. In the development of the larval salivary gland mitotic cell division ceases during embryogenesis (14). The larval cells and, in particular, their nuclear structures become extremely large. Because of their size the cytochemist finds in this cell an ideal object with which to work.

Heretofore the salivary gland has been studied principally from the standpoint of its chromosomes. These studies have made use of the salivary gland chromosome as a cytogenetic tool and as an object for the study of chromosomes per se. The cell as a growing, differentiating, and functioning system has been almost entirely neglected. However, the salivary gland cell, as an active and rapidly changing physiological unit is the primary interest of the present investigation.

What will essentially be presented in this paper is an account of the nucleic acid localizations during development showing their possible relationship to the growth and functional activities of the salivary gland cell.

MATERIALS AND METHODS

The observations to be described are based on a study of some 200 salivary gland pairs from larvae of *Drosophila robusta* ranging in age from hatching up to 36 hours after puparium formation. The laboratory stock used was derived by Max Levitan of Columbia University and is homozygous for all standard gene arrangements. Except for a small amount of unanalyzed male sterility (4), it is highly vigorous.

The larvae were raised in shell vials containing standard cornmeal-Karo-agar slants inoculated with fresh yeast. The larvae were reared at a temperature of 25° C

[1] Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Zoology, Washington University, St. Louis, Missouri.

± 1.0° C. Optimal food conditions were maintained at all times. The life span of *D. robusta* larvae are about twice that of *D. melanogaster*, being $9\,^1/_2$ to 10 days. All larval ages were calculated in hours from oviposition. The times of moulting and puparium formation vary considerably even among larval sibs. This variation in time of development was minimized by using young fast-laying females, collecting eggs at closely-timed intervals, and discarding all late or early hatched larvae.

In order to obtain salivary gland preparations in which the cells could be compared as to size and as to intensity of staining reaction, a uniform schedule of fixation, dehydration, and embedding was adhered to in the preparation of all tissues. Carnoy's fixative, 3 parts absolute alcohol to 1 part glacial acetic acid, was used throughout. Although the salivary gland tissues are quite delicate, it was found that cellular distortion could be cut to a minimum by reducing the time in fixation (30 minutes), washing in 95 per cent ethyl alcohol, and clearing in amyl acetate. As a clearing agent amyl acetate is ideal since it is miscible in both 95 per cent alcohol and paraffin (7).

Three techniques were utilized for the localization and identification of the two nucleic acids: basic staining with toluidine blue, Brachet's ribonuclease technique, and the Feulgen reaction.

The toluidine blue staining procedure used was a modification of the procedure described by Brachet. Tertiary butyl alcohol was used for de-staining since unlike ethyl alcohol it removes only that dye which is not chemically combined with the components of the cell (12). Toluidine blue is non-specific, however, in the sense that it does not distinguish between nucleic acids but stains both DNA and RNA equally well. In Carnoy-fixed tissues only proteins and nucleic acids remain in significant quantities in the cell (11). Of these, toluidine blue stains only the basophilic nucleic acid component.

A modification of Brachet's (3) ribonuclease method established the identity and localization of the ribonucleic acid (6). Two slides of a series were de-paraffinized and hydrated through a graded series of alcohol to water. One slide was placed in a Coplin jar containing 0.2 per cent ribonuclease (0.2 mg/ml) in distilled water, the other slide which served as a control was placed in distilled water. Both slides were incubated at 40° C for one hour. After the end of the incubation period the slides were washed for five minutes in running tap water to completely remove the soluble products of the enzyme hydrolyzation. The control and the treated slide were then simultaneously stained in toluidine blue; the same procedure as outlined in the preceding paragraph was utilized.

For demonstrating the presence of desoxyribonucleic acid, the Feulgen method as modified by de Tomasi (16) and by Stowell (15) was followed closely. According to Bauer (2) and Hillary (8) the time and temperature of the hydrolysis to be used in order to obtain the maximum reaction varies with the type of fixative and characteristics of the tissue. A series of Carnoy-fixed salivary gland sections showed that the desoxyribonucleic acid of the salivary gland chromosomes gave a maximum Feulgen reaction after five minutes hydrolysis at 50° C.

The stains used in this investigation were: toluidine blue 0, certification number NU8; basic fuchsin, certification number NF56. Toluidine blue and basic fuchsin were obtained from the National Aniline Company. The ribonuclease was prepared according to the McDonald method by the Worthington Biochemical Laboratories.

OBSERVATIONS

The observations discussed here are based on a comparison and analysis of the intensity of the basic toluidine blue staining reaction. The ribo- and desoxyribonucleic acid localizations were previously determined by the use of the ribonuclease technique and the Feulgen reaction.

Riobonucleic acid

The salivary gland cytoplasm is highly basophilic through the first and second stages and up to the mid-point of the third at which time there begins a decided decrease in basophilia. During the third instar the gland cells increase rapidly in size. This size increase is due partially to growth as a result of polytenic division and partially to the piling up in the cytoplasm of the deutoplasmic substances.

The principal source of nuclear ribonucleic acid is to be found in the nucleolus. The nucleolus of the salivary gland nucleus is an amazing structure in that unlike the nucleoli of normal mitotic cells it increases in size rather than number as the chromosomes become more polytene. For this reason the compound nucleolus of the giant third instar nucleus becomes exceedingly large, e.g., it exceeds in dimensions the entire nucleus of most mammalian tissues. The even-staining, highly-basophilic first instar nucleolus soon takes on a "vacuolated" appearance which is characteristic of the nucleoli of the remaining stages. The variability of the nucleolar basophilia and the irregularity of its shape is extreme. The most striking feature of this nucleolus is the light-staining well defined areas which from their general morphology suggest liquid-filled "vacuoles," hence the term "vacuolated." These "vacuoles" are more common in the nucleoli of actively secreting cells in which the cytoplasm is highly basophilic. Beyond the mid-point of the third instar the nucleolus becomes reduced in basophilia and noticeably less "vacuolated."

Desoxyribonucleic acid

The chromosomes of the first instar nucleus are scarcely visible threads lying in a spiraled ring around the nucleolus close against the nuclear wall. The characteristic banded structure of the salivary gland chromosome does not become visible until early in the third instar. Obviously the absence of visible bands does not mean that they are not polytene; they undoubtedly

became polytene early in the first instar. Desoxyribonucleic acid is confined to the bands. No Feulgen positive ring could be detected around any of the nucleoli. The basophilic chromocenter is for the most part ribonucleic acid; however, very faint Feulgen positive threads were detected connecting the chromosome arms and apparently tying the nucleolus to the chromocenter.

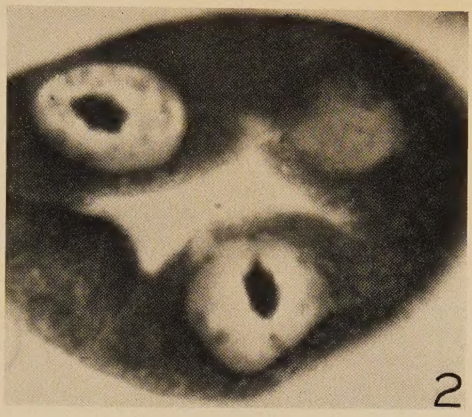
The nucleic acids during metamorphosis

The formation of the puparium case marks the beginning of metamorphosis. However, the larval gland frequently shows signs of disintegration before the pupa case actually forms. The autolytic process once begun progresses rapidly. From the manner in which the cytoplasm disintegrates, it would appear to be formed of two distinctly different substances: one which diffuses rapidly out of the cell soon after puparium formation and another which forms the structural framework of the cell and remains behind. The former substances are the non-nucleic acid deutoplasmic substances which will be discussed with the secretory phase. The latter more stable substance contains the nucleic acids, both RNA and DNA. The ribonucleic acid of the cytoplasm condenses into dark staining strands in the early stages of autolysis. The last substances to disintegrate are those which make up the cell walls, the nuclear walls, and the chromosome framework.

There is no cytological evidence that the nucleoli of *D. robusta* undergo fragmentation as reported by Painter (13) for *D. melanogaster*. They do, however, become increasingly irregular in shape from the midpoint of the third instar and seem to decrease rapidly in size after the gland ceases to function as a secretory organ. It is possible to positively identify the nucleolus as a discrete structure even after the gland has literally fallen apart and all that remains is a mass of cell debris.

The bands of the chromosomes stain intensely but do not maintain their original continuity in that several bands fuse forming heavy dark clumps. This reaction of chromosomes from these distintegrating cells is closely akin to Darlington's (5) "sticky" chromosomes which he observed in irradiated cells.

The other larval tissues are also of interest cytochemically. Many of these cells, found in the ducts, the fat, the malpighian tubules, and the gut, are similar at hatching to the gland cell both as to size and as to degree of cytoplasmic basophilia. All these cells increase in size during the course of larval development, but their rate of growth lags behind that of the salivary gland. The chromosomes become polytene, except in the fat cells where



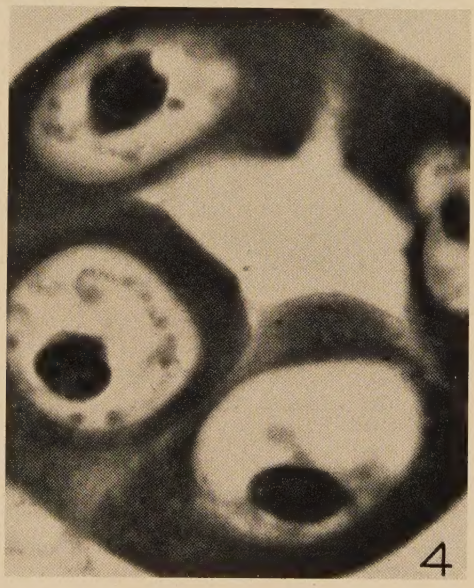


Fig. 1. This section is cut through the entire larva (1st instar) at the level of the salivary gland. The cytoplasm appears to be quite homogeneous and highly basophilic. The inner cell border is very diffused. There are no chromosomes visible in this photomicrograph, however, by careful focusing they can be identified as slender threads near the nuclear membrane. The nucleoli are large and irregular.

Fig. 2. A section through the gland of a mid-second instar larva (125 hours). The cytoplasm is becoming somewhat granular in nature. The chromosomes are clearly visible but are highly coiled and not visibly banded.

Fig. 3. A gland section from a larva (136 hours) in the process of moulting from the second instar to the third instar.

Fig. 4. The gland of an early third instar larva. The chromosomes are distinctly banded. The nuclei and nucleoli are increasing rapidly in size at this stage of development.

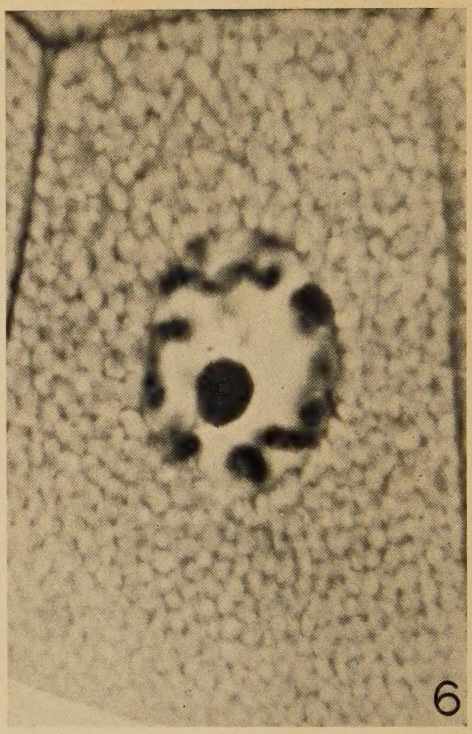


Fig. 5. The gland of a mid-third instar larva. The cytoplasm is becoming distended with the deutoplasmic substances and has, as a consequence, taken on a reticulated appearance. The inner lumen cell border is well formed and regular while the lumen, itself, is much reduced. The nucleoli reach their maximum size at this stage.

Fig. 6. A section taken from the gland of a very late third instar larva just prior to puparium formation. The chromosomes, at this stage of the third instar, reach their highest degree of polytenic development. The cytoplasm appears to be much reduced in basophilia. The total amount of RNA may however still be the same but only stretched-out over a greater area by the accumulation of the cytoplasmic inclusions. The nucleoli are becoming much smaller.

All photomicrographs were taken on Eastman Verichrome film with an Exacta, Model B, and 60 mm extension tube, using an apochromatic objective (X 90). All figures × 1000.

they appear to be polyploid, but never reach the high degree of polyteny attained by the gland cell. The cytoplasm, of all the above type cells, remains densely homogenous and intensely basophilic throughout larval development. At metamorphosis these tissues disintegrate after the salivary gland.

Secretory function

Painter (13) and Hsu (9) were among the first to postulate a secretory function for the larval salivary gland of *Drosophila*. Hsu in his study of the Golgi and mitochondrial materials of the gland cell contributed convincing cytological evidence to support his contention. The secretory mechanism, as described by Hsu, resembles the merocrine type in that the cells maintain their identity during the cyclic secretory process. The cytological evidence depends upon the irregular appearance of the inner cell border and the presence of stainable substances within the lumen.

The first definite sign of secretory action was found in the larval gland of the late first instar. Figure 1 shows the diffused inner border of cells that have discharged their contents into the gland lumen and are in the process of repair. The gland is highly active through the entire second instar and the first half of the third. The ragged inner cell border is clearly visible in figures 1 through 4. In figure 5 (mid-third instar larva) the lumen has become regular in contour and much reduced in size. From this stage up to the beginning of metamorphosis the gland is no longer active as a secretory organ. It is also during this period that the cytoplasm becomes more "vacuolated" and less basophilic.

Little is known concerning the chemical nature of the secretion substance although Kodani (10) analyzed the protein component. He found virtually no nucleic acids; however, he was able to identify several amino acids and some inorganic salts. In the present study basophilic substances were usually found in the lumen of the active gland indicating that the secretion substance may very well contain some ribonucleic acid (Feulgen negative, removed by ribonuclease).

As to the chemical nature, source, and possible role of the deutoplasmic substances which accumulate in the cytoplasm, one can only speculate. Painter (13) suggests that they are secretion substances which are piling up in the cell prior to secretion. However, the gland cells which Painter describes as being actively secreting are similar, as a comparison of his figure 17 to figure 6 of this paper will show, to the cells of a late third instar larva. Glands of this age larva are not active secretory organs. Furthermore, Painter's figure 21 is plainly that of a degenerating cell and his secretory "globules" are the result of autolyzing cytoplasm.

Hsu postulates a dual role for the salivary gland of *Drosophila* larvae, i.e., secretion and storage of food substances. Hsu's food substances, Painter's secretory globules, and the so-called deutoplasmic substances described here are obviously the same. These cytoplasmic droplets increase in number as the process of autolysis progresses and in advance stages of autolysis the droplets coalesce to form huge globules. Since they do not stain with either toluidine blue or fast green it can be assumed that they contain no nucleic acids or basic proteins.

An examination of unfixed cells of similarly aged glands, observed in vitally-stained, hanging-drop preparations and with phase contrast, shows the same cytoplasmic formations as found in the fixed preparations. When these cells are treated with Janus Green B (1/10 000) or Neutral Red (1/15000) it was found that the droplets showed a marked affinity for the latter but would not react with the former. This suggests (1) that these cytoplasmic droplets may be lipoid in nature.

DISCUSSION

The salivary gland cell brings together two problems, namely, development and secretion. Both problems are to a certain extent the same, that is, they both involve protein synthesis.

The cytological observations suggest that the gland is functional as a secretory organ up to the mid-point of the third instar. It is during this period that the cytoplasmic basophilia (RNA) is most intense. When secretion ceases there is a simultaneous decrease in RNA concentration. This implies that RNA is directly concerned with secretion. This does not mean that RNA is not concerned in the synthesis of the structural proteins needed by the growing cell but rather that the demands of the secretory function overshadow the demands for growth.

SUMMARY

The localization and identification of the two nucleic acids, ribonucleic and desoxyribonucleic, have been determined for the larval salivary gland of *Drosophila robusta*. The determinations were made by the use of basic staining, Brachet's ribonuclease method, and the Feulgen reaction.

The cytological observations suggest that the gland is functional as a secretory organ up to the mid-point of the third instar. It is during this period that the cytoplasmic basophilia (RNA) is most intense. When secretion ceases there is a decided decrease in RNA concentration. These findings suggest that the RNA system is concerned primarily with secretion.

ACKNOWLEDGEMENTS

I wish to express to Dr. Hampton L. Carson my appreciation for the suggestion of the problem, and for his invaluable advice and constructive criticism during the course of the investigation. I am also indebted to Dr. Ernst J. Dornfeld of Oregon State College for his advice and for Mr. Paul Wilkinson's assistance in the taking of the photomicrographs.

REFERENCES

1. Baker, J. R., *Quart. J. Micro. Sci.*, **90**, 293 (1949).
2. Bauer, N., *Z. Zellforsch.*, **15**, 225 (1932).
3. Brachet, J., *Arch. Biol.*, **44**, 519 (1933).
4. Carson, H. L., and Stalker, H. D., *Evolution*, **1**, 113 (1947).
5. Darlington, C. D., *Symp. Soc. Exptl. Biol.*, **1**, 252 (1947).
6. Dempsey, E. W., and Wislocki, O. B., *Phys. Rev.*, **26**, 1 (1946).
7. Drury, A. F., *Stain. Technol.*, **16**, 21 (1941).
8. Hillary, B. B., *Bot. Gaz.*, **101**, 276 (1939).
9. Hsu, W. Siang, *Quart. J. Micro. Sci.*, **89**, 401 (1948).
10. Kodani, Masuo, *Proc. Natl. Acad. Sci.*, **34**, 131 (1948).
11. Leuchtenberger, Cecilie, *Chromosoma*, **3**, 450 (1950).
12. Michaelis, L., *Cold Spring Harbor Symp. Quant. Biol.*, **9**, 55 (1947).
13. Painter, T. S., *J. Exptl. Zool.*, **100**, 523 (1945).
14. Poulson, D. F., *Actualités sci. et. ind.*, **498**, 1 (1937).
15. Stowell, R. E., *Stain Technol.*, **20**, 45 (1945).
16. de Tomasi, J. A., *Ibid.*, **11**, 137 (1936).

STUDIES ON THE SALIVARY GLAND OF *DROSOPHILA*

II. CHANGES IN NUCLEAR AND NUCLEOLAR VOLUMES AND THEIR POSSIBLE SIGNIFICANCE

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It is well known that the growth of many organs of insect larvae is not accompanied by the usual form of mitotic proliferation but by an increase in cell size only (6, 7). In the salivary gland this increase is accompanied by corresponding changes in nuclear size and an increase in size of the chromosomes and the nucleolus. Each of these nuclear structures remain in a permanently coalesced condition during larval development and reach a giant size in the third instar. It was observed that the nucleolus of the salivary gland of *Drosophila* is, in addition, extremely variable during larval development. However, this variability appears to fluctuate with the metabolic condition of the cell. Recent studies on the nucleic acids of a wide variety of cells (1, 3, 4, 5, 8) suggest that the nucleolus is intimately involved in nucleic acid metabolism. The nucleolar behavior has been found to be particularly striking in all cells and tissues active in protein synthesis, e.g., embryonic, secretory, tumor, etc.

METHODS

The larval salivary glands were fixed in Bouin, sectioned at 10 microns, and stained with toluidine blue. Measurements of nuclei and nucleoli were made on a closely graded series of the above gland cells. Since the glands of all age larvae were handled in the same manner the results can be considered comparable.

The nuclei and nucleoli of the very early first instar gland cells are spherical but as they undergo repeated polytenic divisions the shape becomes ellipsoidal. Measurements of nuclei and nucleoli of each stage of larval development were averaged and volumes calculated (Table I). Later measurements made on unfixed cells agreed closely with those made on Bouin or Carnoy (3: 1) fixed cells.

DISCUSSION

The increase in nucleolar volume follows the same general pattern of increase as that of the nucleus. The former, however, reaches its volumetric peak at the mid-point of the third instar (approximately 170 hours after

TABLE I

Nuclear and Nucleolar Volumes

Ages in hours after oviposition		Nuclear volumes microns cubed	Nucleolar volumes microns cubed
50	(7)[1]	38	2
60	(3)	78	5
90	(5)	129	7
115	(12)	288	65
125	(3)	546	73
135	(5)	2 136	140
145	(11)	4 943	401
150	(8)	5 362	439
160	(5)	6 576	670
170	(6)	8 713	1 273
180	(7)	13 094	490
185	(3)	15 846	452
190	(7)	12 700	229
12[2]	(7)	7 810	158
24	(5)	4 760	153

oviposition) and begins to decrease shortly thereafter. The nucleus continues increasing in volume until near the end of the third instar (approximately 190 hours) at which time it likewise begins to decrease in size. The nucleus declines just prior to puparium formation. The decrease in nucleolar size is accompanied by a loss of the so-called "vacuoles" and by the taking-on of an increasingly irregular shape. The loss of nuclear volume, coming as it does after the start of metamorphosis, is probably due entirely to autolysis. Nuclear size, as would be expected, directly reflects the degree of chromosome polyteny; i.e., the number of times the gene chain has been duplicated.

The loss of nucleolar volume cannot be so simply explained since metamorphosis has not as yet begun. The nucleolar changes parallel the secretory behavior (5) of the gland and the cytoplasmic basophilia of its cells. It would seem, therefore, that there must exist a close relationship between the ribonucleic acid system, the nucleolus and the secretory function of the cell. The nucleolus is very probably concerned in both growth and secretion (1), however, in the case of the drosophilan salivary gland the demands of the latter far exceed those of the former.

[1] Total number measurements 576 on 94 different glands at 15 different age levels.

[2] Hours after puparium formation.

Volumes calculated from the average of the measurements of a minimum of 10 nuclei and nucleoli from each gland studied.

SUMMARY

An analysis of the nuclear and nucleolar volumes suggests: 1) There is a close correlation between cytoplasmic basophilia and nucleolar size, 2) the nucleolus and the RNA system is directly concerned with secretion and 3) there appears to be an equally direct connection between nuclear size and the degree of chromosome polyteny.

REFERENCES

1. Caspersson, T., *Symposia Soc. Exptl. Biol.*, **1**, 127 (1947).
2. —— Cell growth and function. Norton & Co. pp. 185, 1950.
3. Darlington, C. D., *Symposia Soc. Exptl. Biol.*, **1**, 252 (1947).
4. Koller, P. C., *ibid.*, **1**, 270 (1947).
5. Lesher, S., *Exptl. Cell Res.*, **2**, 577 (1951).
6. Trager, W., *J. Exptl. Zool.*, **71**, **489** (1936).
7. —— *ibid.*, **76**, 467 (1937).
8. Yokoyama, Hisako O., and Stowell, Robert E. In press (1951).

TRANSFORMATIONS ALLOGÈNES DU PNEUMOCOQUE

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avec la collaboration technique de Mme. L. GOLDSTEIN

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Reçu le 3 Février, 1951

Lorsqu'on fait agir, dans des conditions qui seront rappelées plus bas, un extrait de pneumocoques à capsule sur une culture de pneumocoques sans capsule, on y induit l'apparition de bactéries semblables à celles de la souche utilisée pour la préparation de l'extrait. Ces expériences, réalisées par Griffith (4) et Avery, MacLeod et McCarty (1), montrent que chaque type de pneumocoque à capsule renferme un agent responsable de la formation et de la spécificité du polysaccharide capsulaire. L'étude chimique de ces agents (« transforming principles » ou « TP » de Avery) indique qu'ils sont composés uniquement d'acide désoxyribonucléique (ADN). Leurs propriétés biologiques suggèrent d'autre part qu'ils représentent des unités génétiques, responsables de leur propre propagation dans la bactérie, aussi bien que de la formation d'un polysaccharide capsulaire spécifique. On reconnaît, à l'heure actuelle, plus de 70 types différents d'antigènes capsulaires et il faut supposer qu'il y a autant de TP spécifiques, bien qu'on n'en ait isolé que trois sous forme purifiée (11).

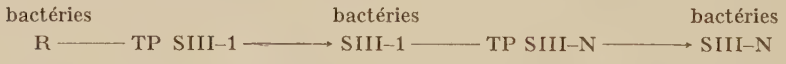
La diversité des types de pneumocoques peut être attribuée à des mutations du TP au cours de l'évolution. Cependant on n'a jamais enregistré dans les conditions expérimentales l'apparition d'une mutation conduisant à la formation d'un nouveau polysaccharide.

Les nombreux mutants observés chez les pneumocoques de plusieurs types sont caractérisés par des modifications *quantitatives*. Des mutants à capsule réduite on a pu extraire des TP capables d'induire chez les pneumocoques sans capsule la formation d'une capsule semblable à celle de la souche qui a fourni le TP. Chacun de ces mutants contient donc un TP muté. Quoique ces mutations du TP ne se manifestent au niveau du phénotype que par des changements apparemment quantitatifs, tout changement de l'activité biologique d'un TP relève nécessairement d'une altération de sa structure dont les détails inconnus seraient intéressants à connaître. Ce sont ces considérations qui nous ont conduit à poursuivre l'étude des TP mutés commencée il y a quelques années et dont, en guise d'introduction, il est utile de rappeler ici l'essentiel.

Notre étude porte sur des mutants du pneumocoque du Type III. Tous les mutants étudiés appartiennent à l'une de deux classes distinctes, caractérisées par le degré de réduction de la synthèse capsulaire (15). Nous les appellerons SIII-1 et SIII-2 (voir tableau I). La capsule des bactéries de la classe SIII-1 est si réduite que celles-ci ne se comportent pas, dans les réactions sérologiques classiques, comme des bactéries à capsule typiques. Les bactéries SIII-2 possèdent au contraire une véritable capsule, mais celle-ci est encore considérablement réduite par comparaison avec la capsule des pneumocoques normaux (SIII-N).

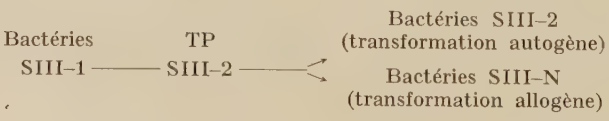
La présence d'une capsule complète semble constituer une entrave à la transformation d'une bactérie. C'est pourquoi nous ne pourrons étudier que les transformations des pneumocoques sans capsule (R) et des pneumocoques SIII-1 qui ne secrètent qu'une capsule rudimentaire.

Il est possible de réaliser en deux étapes la transformation suivante:



et on peut montrer que les bactéries SIII-N ainsi produites ne renferment que l'agent capsulaire SIII-N. Ceci conduit à la conclusion qu'un pneumocoque ne peut contenir qu'un seul TP capsulaire, et que l'agent SIII-1 est remplacé au cours de la deuxième étape par l'agent normal, inducteur de la deuxième transformation (15).

Tandis que le phénomène décrit suggère que la transformation consiste en un remplacement de l'agent contenu dans la bactérie par l'agent introduit sous forme purifiée dans le milieu de culture, les résultats d'autres expériences ne sont pas compatibles avec une telle interprétation. Ainsi, la souche SIII-1 transformée par le TP de la souche SIII-2 donne deux sortes de bactéries: des pneumocoques SIII-2, entièrement semblables aux bactéries qui ont fourni le TP, et des pneumocoques SIII-N, à capsule normale. Ces derniers ont un phénotype qui ne correspond ni au TP de la souche soumise à l'action du TP, ni à celui de la souche ayant fourni celui-ci. On peut donc distinguer dans ce cas deux sortes d'activités du TP SIII-2: une *action autogène*, qui donne naissance aux bactéries SIII-2, et une *action allogène*, qui est à l'orgine des bactéries à phénotype nouveau. Cette double transformation peut être représentée par le schéma suivant:



L'activité autogène peut bien s'expliquer par un simple remplacement du TP SIII-1 par le TP SIII-2. L'activité allogène requiert au contraire une autre explication et nous a conduit à supposer que les transformations allogènes sont dues à la reconstitution d'un agent transformant normal à partir des deux agents mutés: celui se trouvant dans les pneumocoques (SIII-1) et celui introduit dans le milieu de culture (SIII-2).

On verra que le présent travail, qui porte sur plusieurs sortes de transformations allogènes, fournit des données en faveur d'une telle interprétation et montre que les nouveaux agents, résultats des transformations allogènes, peuvent être soit normaux, soit mutés, selon les propriétés spécifiques des deux agents impliqués dans leurs formation.

MATÉRIEL ET MÉTHODES

Souches de pneumocoques. La plupart des souches employées dans ce travail sont les mêmes que celles qui ont servi dans les études des transformations induites réalisées au laboratoire de Avery, à l'Institut Rockefeller de New York. Certaines autres sont des lignées mutantes isolées à partir des souches de Avery. La liste complète comporte, outre les races indiquées dans le tableau I, la souche R36A, lignée « rough »

Tableau I

Liste des souches de Pneumocoques normaux et mutés Type III.

	Phénotype		
	SIII-1 (mutée)	SIII-2 (mutée)	SIII-N (normale)
Morphologie coloniale	« rough »	« smooth »	« smooth » (mucoïde)
Formation de précipitine du type III	+	+ + +	+ + + +
Réaction de Neufeld	—	+	+
Antigènes somatiques	exposés	couverts	couverts
Souches	SIII-1[3] SIII-1a[1] SIII-1b[1] SIII-1c[2]	SIII-1[3]	A66 SV-III

Origine des mutants:
[1] Mutation spontanée de A66.
[2] Mutation spontanée de SV-III.
[3] Mutation spontanée de l'agent transformant de la souche A66, pendant ou après la transformation de la souche sans capsule, R36A (voir Taylor, 1949).

dérivée de la souche à capsule du Type II, D39. On trouvera dans le tableau l'origine de chaque souche, aussi bien que ses propriétés sérologiques. En ce qui concerne ces dernières, on se rappellera que les souches qui ont une véritable capsule ne sont pas agglutinées par les anticorps dirigés contre les antigènes somatiques. Ainsi, les souches SIII-N et SIII-2 ne sont agglutinées que par des anticorps contre le polysaccharide capsulaire du Type III. Les souches de la catégorie SIII-1 sont, au contraire, agglutinées par des immunserums anti-rough ou anti-Type III smooth, ce qui indique que la surface de ces bactéries n'est pas entièrement couverte par une couche de polysaccharide capsulaire. On se rappellera d'autre part que le polysaccharide capsulaire des pneumocoques est soluble dans l'eau. En conséquence, l'agglutination des souches qui en font une sécrétion active n'a lieu qu'en présence de grandes quantités d'anticorps spécifiques, une partie de ces derniers étant neutralisée par le polysaccharide en solution. Les titrations d'agglutination des divers mutants montrent que, plus la sécrétion capsulaire est réduite, plus les anticorps capsulaires sont efficaces.

Le groupement de plusieurs souches mutantes dans la même catégorie SIII-1 est justifié par des titrations d'agglutination identiques. Il est bien entendu que l'on ne peut considérer les quantités de polysaccharide formées par les divers mutants comme semblables qu'aux erreurs près de la méthode de titration utilisée. La méthode employée ne permettrait évidemment pas de déceler de très faibles différences entre les quantités de polysaccharides capsulaires de ces diverses souches.

On ignore à l'heure actuelle si les pneumocoques SIII-1 et SIII-2 synthétisent des polysaccharides strictement identiques au polysaccharide du Type III normal et par conséquent si la synthèse de polysaccharide par ces mutants est affectée qualitativement aussi bien que quantitativement. Des données décrites dans une publication antérieure ont rendu peu probable l'existence de différences qualitatives, mais la question ne pourra être considérée comme résolue tant qu'un travail beaucoup plus étendu n'aura pas été effectué. Récemment, nous avons isolé une petite quantité de polysaccharide de la souche SIII-2. Ce polysaccharide permet de précipiter tous les anticorps du Type III d'un immunserum anti-SIII-N. Ce fait exclut l'existence d'une différence de structure entre ce polysaccharide et celui de la souche normale SIII-N, mais n'exclut pas l'existence des différences dans le degré de polymérisation des deux polysaccharides en question.

Milieux. Deux milieux liquides et un milieu solide sont employés dans ces études.

1) Pour isoler les agents transformants à partir des diverses souches, les pneumocoques sont cultivés dans le milieu suivant:

30 g neopeptone (Difco), 12 g d'extrait de levure (Difco), 26 g Cl Na et 3 litres d'eau distillée.

Cette quantité de milieu est répartie dans deux fioles de 3 litres, et stérilisée à 120° C. pendant 30 minutes. Au moment de l'ensemencement des cultures, 1 cm^3 d'une solution stérile de glucose à 25 p. 100 est ajouté à chaque fiole.

2) Pour l'étude de la morphologie coloniale, ce même milieu additionné de gélose sert comme base d'un milieu solide à sang de lapin. La concentration de glucose est doublée. Avant de verser le milieu dans les boîtes Petri, le milieu gélosé fondu est refroidi à 45° C. et additionné de 2,5 p. 100 de sang de lapin défibriné.

3) Pour le maintien des souches et pour les études des transformations, un milieu plus complexe est requis. Ce milieu, également à base de neopeptone et d'extrait de levure, contient de plus un extrait de sérum de bœuf préparé comme suit:

Du sang entier est recueilli dans des bouteilles vaselinées. Après la formation du caillot, le sérum est décanté et additionné d'un volume égal d'eau distillée. Le mélange est chauffé dans un bain-marie à 90° C. pendant 25 à 30 minutes. On sépare alors les protéines coagulées par filtration sur un grand Buchner. Afin de précipiter davantage les protéines dénaturées, le liquide trouble est acidifié avec de l'acide chlorhydrique jusqu'à pH 5. Le précipité qui se forme est séparé par filtration sur du « Filter Cel » (kaolin). Le pH est ensuite ramené à 7–7.2. L'extrait est réparti dans des flacons et stérilisé pendant 20 minutes à 120° C. Cet extrait peut être gardé au moins un an sans perdre son activité.

Le milieu 3 a la composition suivante:

100 cc extrait de sérum de bœuf, 10 g neopeptone, 4,3 g $PO_4\ HNa_2$, 0,26 g $PO_4\ H_2K$, 0,25 g $SO_4\ Mg$, 0,010 g $Cl_2\ Ca$, 3,0 g ClK, 1 litre d'eau distillée.

La solution subit un traitement avec 10 g de charbon, à pH 3. Le pH est ensuite ramené à 7,6 et le milieu est autoclavé pendant 20 minutes à 120° C. Il se forme un précipité qui est enlevé par filtration. Des quantités mesurées du milieu sont distribuées dans des fioles et stérilisées à 120° C. pendant 20 minutes. Il se forme toujours un deuxième précipité qui se dissout au bout de quelques jours. Avant l'emploi, on ajoute, pour 100 cm^3 de milieu, 0,1 cm^3 de glucose à 25 p. 100 stérile et 4 cm^3 d'une solution d'extrait de levure à 10 p. 100, préalablement traitée avec du charbon à pH 3 et stérilisée par filtration.

Dans ce milieu, la population maxima est d'environ 10^8 pneumocoques par cm^3; la durée de génération est de 28 minutes à 37° C. pour la souche R36A. La croissance est limitée par le glucose, dont la concentration ne permet pas la baisse du pH au-dessous de 6,7.

Technique des transformations. Les détails de la technique des transformations ont été décrits antérieurement (15). Rappelons simplement que les transformations ont lieu dans des cultures en pleine croissance, en présence de substances accessoires (12) et d'un agent transformant.

La préparation des agents transformants. La technique de préparation des agents transformants employée ici est essentiellement celle décrite par MacLeod et Krauss (10). Elle consiste à obtenir une très forte croissance dans un volume limité de milieu nutritif et à la faire suivre par la lyse des bactéries dans le milieu de culture même. Après la lyse induite par du désoxycholate de sodium en présence de citrate de sodium (11), l'acide nucléique est précipité par l'alcool. Le précipité est remis en solution et déprotéinisé. L'extrait bactérien déprotéinisé subit alors un fractionnement selon une nouvelle technique qui permet la séparation de la grande partie, sinon de la totalité, de l'acide ribonucléique dans une première fraction, de l'acide désoxyribonucléique dans une seconde et du polysaccharide capsulaire dans une troisième. Les détails de ce fractionnement seront résumés plus bas. Les étapes de la préparation d'un agent transformant seront résumées d'abord.

1) On prépare une culture dans le milieu 1, ensemencé avec des bactéries jeunes ayant proliféré dans le milieu 3 additionné d'un peu de sang de lapin.

2) Le lendemain, on ajoute à cette culture 35 cm^3 de glucose et du rouge de phénol.

TABLEAU II

Propriétés des trois fractions des extraits bactériens déprotéinisés.

	Fraction A	Fraction B	Fraction C
Viscosité	faible	forte	forte
Réactions:			
de Bial	très forte	faible	
de Dische	faible ou absente	forte	faible
avec les anticorps du Type III	négative	positive[1]	très forte[1]
Maximum d'absorption	258 mμ forte	258–260 mμ forte	258–260 mμ faible
Principal constituant	acide ribonucléique	acide désoxyribonucléique	polysaccharide

La croissance reprend et l'acide produit est neutralisé, au fur et à mesure de sa formation, par addition de NaOH 1 N. Au bout de 3 à 4 heures, ce qui correspond à l'addition de 75 cm^3 de soude, la population s'élève à $2{,}5 \times 10^{10}$ bactéries par cm^3.

3) On ajoute alors du citrate de sodium pour rendre le milieu 0,1 normal, puis on induit la lyse par addition de 10 cm^3 d'une solution de désoxycholate de sodium à 10 p. 100 par litre de milieu. La lyse, effectuée à 37° C., est complète en 15 minutes.

4) Aussitôt après la lyse, on ajoute lentement 0,55 volumes d'alcool, tout en agitant le milieu. Quelquefois l'acide nucléique se précipite sous forme de fibres que l'on peut enrouler sur un agitateur. Plus souvent le précipité est recueilli par centrifugation.

5) Le précipité est suspendu dans 100–200 cm^3 de NaCl à 0,85 p. 100 et les protéines sont séparées par la technique de Sevag (13). Cette étape est considérablement facilitée par l'addition à la suspension, au cours des premières extractions, de quelques gouttes de désoxycholate de sodium à 10 p. 100. Après 6 à 10 traitements par le chloroforme et l'alcool amylique on ne peut d'habitude plus extraire de protéines. La solution limpide et visqueuse est alors additionnée de $MgCl_2$ jusqu'à une concentration finale de 1 p. 100 pour effectuer le fractionnement à l'alcool éthylique. Celui-ci est ajouté goutte à goutte pendant qu'on remue vigoureusement.

Lorsque la concentration d'alcool atteint 20 p. 100, la solution se trouble, puis un précipité se forme lentement. Après 24 heures à froid, on sépare celui-ci par centrifugation à 3 000 tours pendant 30 minutes. Il constitue la fraction A.

La fraction suivante est obtenue en portant la concentration d'alcool à 26 p. 100 (0,35 volumes d'alcool ajouté): des fibres se séparent brusquement. On les enroule immédiatement sur un agitateur (Fraction B).

Enfin, à cette même concentration d'alcool, on voit se former plus lentement un

[1] Pour les préparations faites à partir des souches Type III.

précipité floconneux qui correspond à la grande partie du polysaccharide capsulaire (Fraction C). Les vitesses différentes de formation des deux précipités, ainsi que la nature physique différente de ceux-ci, permettent la séparation de l'acide désoxyribonucléique du gros de polysaccharide.

Jusqu'ici les fractionnements ont été pratiqués sur de petites quantités de matériel et l'étude détaillée de la composition de chaque fraction n'a pas été entreprise. Toutes les données recueillies à ce sujet sont résumées dans le tableau II. On peut dire en plus que la fraction A, qui renferme de l'acide ribonucléique, peut être rendue entièrement négative à la réaction de Dische (pour le désoxyribose) par une deuxième précipitation. La forte absorption de l'acide ribonucléique à 258 mμ a permis de constater dans une expérience que 93 p. 100 de la substance absorbante ne dialyse pas. Néanmoins, il est très probable que, contrairement à l'acide désoxyribonucléique, l'acide ribonucléique est dépolymérisé pendant la lyse et on peut se demander si le succès du fractionnement ne repose pas sur cette différence dans l'état de polymérisation.

La méthode de séparation des deux acides nucléiques qui vient d'être décrite est plus douce que les méthodes couramment employées, mais la pureté des deux fractions reste à déterminer (1).

Lorsqu'il s'agit de préparations faites à partir de pneumocoques du Type III, la fraction B, qui contient la presque totalité de l'acide désoxyribonucléique, donne une réaction sérologique due à la présence de polysaccharide capsulaire. On peut la débarasser de toute activité sérologique en répétant le fractionnement 4 ou 5 fois, mais on perd toujours un peu d'acide désoxyribonucléique. C'est pourquoi nous n'avons d'habitude pas purifié de cette façon les extraits employés au cours du présent travail.

RÉSULTATS EXPÉRIMENTAUX

I.—Les diverses transformations allogènes. Les expériences suivantes ont été entreprises pour savoir si l'interaction entre toute paire de TP mutés peut conduire à une transformation allogène. Des isolements des TP capsulaires ayant été effectués à partir de chacune des souches énumérées dans le tableau I, nous avons testé l'activité de chacun de ces agents sur toutes les souches capables d'être transformées. Le tableau III résume les résultats obtenus. Il en ressort que:

1) Une souche ne donne jamais de transformation sous l'influence du TP isolé à partir d'elle-même.

2) Plusieurs souches à TP capsulaire muté donnent des transformations allogènes lorsqu'elles sont traitées par un autre TP muté (cas indiqués en caractères gras dans le tableau III).

3) Les TP SIII-1, SIII-1a et SIII-1c, qui proviennent de souches phénotypiquement semblables, ont des activités transformantes nettement différentes et doivent par conséquent être considérés comme différents. Il faut supposer que chacun de ces agents a muté d'une façon particulière.

TABLEAU III

Activité transformante des acides désoxyribonucléiques isolés à partir des souches du Type III sur les diverses souches à phénotype SIII-1 et la souche sans capsule, R36A. Les transformations allogènes sont inscrites en caractères gras. 0 indique l'absence de transformation.

Souche traitée	TP dans les bactéries	Formes induites sous l'action du TP					
		SIII-1	SIII-1a	SIII-1b	SIII-1c	SIII-2	SIII-N
R36A	?	SIII-1	SIII-1a	SIII-1b	SIII-1c	SIII-2	SIII-N
SIII-1	SIII-1	0	**SIII-2**	**SIII-2**	**SIII-N**	SIII-2 et **SIII-N**	SIII-N
SIII-1a	SIII-1a	**SIII-2**	0	0	**SIII-N**	SIII-2 et **SIII-N**	SIII-N
SIII-1b	SIII-1b	**SIII-2**	0	0	**SIII-N**	SIII-2 et **SIII-N**	SIII-N
SIII-1c	SIII-1c	**SIII-N**	**SIII-N**	**SIII-N**	0	SIII-2 et **SIII-N**	SIII-N

4) Seuls les TP SIII-1a et SIII-1b paraissent être identiques au point de vue de leur activité transformante. On peut donc supposer que les souches SIII-1a et SIII-1b contiennent des agents transformants mutés de la même manière. Ceci revient à admettre que la même mutation spontanée a été décelée deux fois.

5) Des transformations réciproques donnent le même résultat. Par exemple, la transformation de la souche SIII-1 par le TP SIII-1a, aussi bien que la transformation de la souche SIII-1a par le TP SIII-1, donnent lieu à l'apparition de pneumocoques à phénotype SIII-2.

6) Le fait que les transformations allogènes ne se limitent pas à la formation de pneumocoques SIII-N est également digne de remarque. Les transformations qui aboutissent à la formation de pneumocoques SIII-2 ne sont que des restitutions partielles du pouvoir de sécrétion du polysaccharide.

7) Enfin, on voit que le TP SIII-2 induit dans toutes les souches SIII-1 à la fois des transformations autogènes (en SIII-2) et des transformations allogènes (en SIII-N). On peut donc se demander si le TP SIII-2 n'a pas

une activité très particulière qui le distingue des TP SIII-1, SIII-1a. Ces derniers induisent en effet, dans les souches SIII-1, seulement des transformations allogènes. Cependant, il est évident que la transformation autogène d'une souche SIII-1 par l'agent d'une autre souche à phénotype SIII-1 ne serait pas décelable par le simple examen de la morphologie coloniale. Il est tout à fait possible que les transformations allogènes sont toujours accompagnées de transformations autogènes, mais que celles-ci échappent à l'observation car le phénotype des bactéries ayant subi cette sorte de transformation se confond avec celui des bactéries non transformées.

Cette première série d'expériences soulève trois questions qui seront abordées maintenant.

II.—Fréquence des transformations allogènes. On peut se demander si les diverses transformations allogènes se produisent avec des fréquences identiques. L'étude précise de ce problème requiert la mise au point de méthodes nouvelles. Cependant, quelques expériences préliminaires montrent dès à présent que les fréquences des diverses transformations allogènes d'une même lignée sont extrêmement différentes et dépendent du TP employé pour induire la transformation.

Dès le début de la présente étude, il nous est paru évident que les transformations allogènes qui donnent naissance aux bactéries SIII-2 sont des événements beaucoup plus rares que toutes les autres transformations rencontrées. Afin de nous en assurer d'une façon plus objective, des titrations de l'activité transformante de deux TP ont été entreprises. Le tableau IV présente les résultats de ces titrations faites dans des conditions standard. Les titrations *A* et *B* ont été effectuées le même jour avec la même culture SIII-1, et la titration *C* quelques jours plus tard. La titration *A* montre qu'au moment de l'expérience, la souche SIII-1 était très susceptible d'être transformée par les TP SIII-1c. La titration *B* indique qu'au même moment cette souche ne donnait que très difficilement des transformations allogènes (en SIII-2) sous l'influence de TP SIII-1a. Ceci ne peut être attribué à l'inactivité du TP SIII-1a, car celui-ci s'est montré très actif dans son action sur une souche différente (SIII-1c) pour donner des transformations en SIII-N (titration *C*). Il apparaît donc que les transformations allogènes en SIII-N sont beaucoup plus fréquentes que celles en SIII-2. Cette conclusion est confirmée par d'autres transformations allogènes en SIII-2.

Ces premiers résultats semi-quantitatifs suggèrent que les fréquences des transformations allogènes dépendent dans une large mesure des propriétés des paires de TP.

TABLEAU IV

Titrations de l'activité transformante des acides désoxyribonucléiques extraits à partir des souches SIII-1c et SIII-1a et agissant sur les souches SIII-1 et SIII-1c. Après 24 heures d'incubation, une fraction de la culture traitée est étalée sur milieu gélosé. Les colonies à phénotype nouveau sont recherchées. 0: absence d'induction. +: les formes induites ne représentent qu'une faible partie de la population. Les cultures témoins incubées sans acide nucléique sont dans tous les cas des cultures pures de la souche ensemencée.

	Souche traitée	Tests quintuples	µg d'ADN de la souche SIII-1c par cm³ 0,1	0,01	0,0033	0,001	0,00033
		a	SIII-N	SIII-N	SIII-N	SIII-N$^+$	SIII-N$^+$
		b	SIII-N	SIII-N	SIII-N	SIII-N$^+$	0
A	SIII-1	c	SIII-N	SIII-N	SIII-N	SIII-N	0
		d	SIII-N	SIII-N	SIII-N	SIII-N$^+$	0
		e	SIII-N	SIII-N	SIII-N	SIII-N	0

			µg d'ADN de la souche SIII-1a par cm³ 28	2,8	0,28	0,028	0,0093	0,0028	0,00093
		a	0	0					
		b	0	0					
B	SIII-1	c	0	0					
		d	0	0					
		e	SIII-2$^+$	0					
		a			SIII-N	SIII-N	SIII-N	0	SIII-N
		b			SIII-N	SIII-N	SIII-N	0	SIII-N
C	SIII-1c	c			SIII-N	SIII-N	SIII-N	SIII-N	0
		d			SIII-N	SIII-N	SIII-N	SIII-N	0
		e			SIII-N	SIII-N	SIII-N	SIII-N	0

III.—Non-identité des TP SIII-2 et SIII-2a. On pouvait se demander si des bactéries à phénotype SIII-2 apparues au cours d'une transformation allogène sont identiques avec les bactéries de la souche SIII-2 qui doit son origine à une mutation spontanée.

Nous avons étudié l'agglutination, par les anticorps du Type III, de deux clones, SIII-2a et SIII-2b provenant de transformations allogènes, et de la souche SIII-2 originale. Les résultats des titrations d'agglutination donnés dans le tableau V indiquent que les trois souches comparées secrètent approximativement la même quantité de polysaccharide capsulaire. Aucune de ces souches n'est agglutinée par un immunserum antirough.

TABLEAU V

Agglutination des souches SIII-2, SIII-2a et SIII-2b par un immunserum du Type III. 10^3 bactéries sont ensemencées dans le milieu nutritif qui renferme la quantité indiquée de sérum. Période d'incubation: 20 heures. La souche SIII-2a provient d'une transformation de la souche SIII-1a par le TP SIII-1; la souche SIII-2b d'une transformation de la lignée SIII-1b par le même agent transformant.

Souche ensemencée	Dilution de l'immunserum de cheval, anti-Type III							
	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	Témoin
SIII-2	+ + +	+ + +	+ +	+	—	—	—	—
SIII-2a	+ + +	+ + +	+ +	+	—	—	—	—
SIII-2b	+ + +	+ + +	+ +	+	—	—	—	—

+ + +: les bactéries sont presque entièrement sédimentées.
+ +: petits sédiments, grumeaux en suspension.
+: grumeaux en suspension.
—: pas d'agglutination.

TABLEAU VI

Test qualitatif de l'activité transformante d'un extrait de bactéries SIII-2a sur les souches SIII-1, SIII-1a, SIII-1b et SIII-1c.

Souche ensemencée	TP	Tests quadruples				Témoin sans TP
		1	2	3	4	
SIII-1	SIII-2a	SIII-2	SIII-2	SIII-2	SIII-2	SIII-1
SIII-1a		SIII-2	SIII-2	SIII-2	SIII-2	SIII-1a
SIII-1b		SIII-2	SIII-2	SIII-2	SIII-2	SIII-1b
SIII-1c		SIII-2 SIII-N	SIII-2 SIII-N	SIII-2 SIII-N	SIII-2 SIII-N	SIII-1c

Les méthodes d'agglutination ne permettent donc pas une différentiation de ces lignées.

Toujours en vue de trouver des différences entre les souches SIII-2 et SIII-2a, nous avons étudié l'activité du TP isolé de cette dernière. On se souvient que la transformation des souches SIII-2 n'a jamais pu être effectuée (14, 15). Par conséquent, un test direct de l'activité transformante du TP SIII-2a sur des pneumocoques SIII-2 n'a pas pu être fait. Des expériences de transformation à l'aide de ce TP de bactéries de la classe SIII-1 montrent que le TP SIII-2a diffère du TP SIII-2. Le TP SIII-2 induit dans toutes les lignées à phénotype SIII-1 à la fois des transformations allogènes et autogènes. Or, comme le montre le tableau VI, le TP SIII-2a n'induit

des transformations allogènes que dans la souche SIII-1c. Dans toutes les autres, son activité se borne aux transformations autogènes.

Ainsi, le TP SIII-2a est distinct du TP SIII-2 et on peut se demander si ses propriétés ne reflètent pas son origine. La lignée SIII-2a provient d'une transformation de la souche SIII-1a par le TP SIII-1. Contiendrait-elle les TP de ces deux lignées? Ceci ne nous paraît pas très probable pour les raisons suivantes:

1) En agissant sur la lignée R36A, le TP SIII-2a induit l'apparition de bactéries SIII-2 avec une fréquence relativement grande. Or, s'il était un mélange des TP SIII-1 et SIII-1a, les transformations en SIII-2 devraient être extrêmement rares, car elles requerraient l'intervention simultanée de ces deux TP.

2) De même, en agissant sur les lignées SIII-1, SIII-1a et SIII-1b, ce TP induit constamment des transformations en SIII-2. Or; si le TP SIII-2 était un mélange des TP SIII-1 et SIII-1a, ces transformations devraient être très rares, comme toutes les transformations allogènes en SIII-2.

3) En agissant sur la lignée SIII-1c, le TP SIII-2a induit constamment des transformations en SIII-2 et SIII-N. Or, si cet agent était un mélange des TP SIII-1 et SIII-1a, un tel résultat serait très improbable; chacun de ces TP, lorsqu'ils agissent sur SIII-1c, induisent en effet des transformations en SIII-N. On s'attendrait donc plutôt à ce qu'un mélange des TP SIII-1 et SIII-1a induise également des transformations en SIII-N lorsqu'il agit sur SIII-1c.

Il paraît par conséquent plus probable qu'au cours de la transformation qui a donné naissance à la souche SIII-2a, il s'est formé un nouvel agent transformant (SIII-2a).

IV.—Nature de la transformation allogène en SIII-N. La formation, dans certaines transformations allogènes, de bactéries à phénotype SIII-N, pose des problèmes tout à fait analogues à ceux posés par l'origine de la souche SIII-2a. Ici encore le phénotype SIII-N pourrait être dû soit à ce que les bactéries SIII-N renferment deux TP mutés dont l'action combinée rétablit la sécrétion normale de polysaccharide, soit à ce qu'un agent capsulaire SIII-N a été reconstitué à partir de deux agents mutés. Quoique la première hypothèse paraisse peu probable à la suite de l'étude de l'agent SIII-2a, nous avons, à titre de vérification, étudié l'agent capsulaire d'une lignée SIII-N résultant d'une transformation allogène.

Une lignée SIII-N apparue dans une expérience de transformation de la souche SIII-1a par l'agent transformant SIII-2 a été choisie pour cette étude.

Ce choix a été gouverné par les considérations suivantes. Les bactéries SIII-2 forment des colonies très nettement distinctes des colonies R ou SIII-1. Par conséquent, la présence de l'agent SIII-2 dans l'acide nucléique de la souche allogène SIII-N serait facilement décelée sans l'application de techniques spéciales.

Un clone de bactérie SIII-1a a été traité par le TP SIII-2. Comme toujours, ce traitement a abouti à la formation de pneumocoques SIII-2 et SIII-N. Une colonie SIII-N a été isolée et a fondé le clone qui a servi à l'isolement de l'acide désoxyribonucléique. L'activité transformante de cette substance a été testée sur les souches R36A et SIII-1. Des pneumocoques SIII-2 ne sont apparues dans aucun cas. La préparation utilisée s'est donc comportée comme toute préparation obtenue à partir de pneumocoques SIII-N. Elle paraît par conséquent renfermer seulement un agent transformant capsulaire normal.

Ainsi, les résultats de nos expériences suggèrent que les transformations allogènes résultent de la reconstitution d'un agent capsulaire normal ou muté à partir de deux agents capsulaires mutés différents, le degré de reconstitution dépendant des propriétés spécifiques des deux agents qui ont participé à la reconstitution.

DISCUSSION

On sait que la réduction de la capsule chez certains mutants du pneumocoque est le résultat de la mutation spontanée de l'agent capsulaire (TP) qu'ils contiennent (10, 14, 15). Nous venons de montrer que, lorsque des pneumocoques qui contiennent un TP muté sont traités par un TP muté différent, on observe des transformations allogènes, c'est-à-dire l'apparition de bactéries à synthèse capsulaire entièrement ou partiellement rétablie, selon la paire de TP confrontés. Les expériences qui viennent d'être décrites montrent que cette restitution intéresse non seulement le phénotype de la bactérie, mais aussi son TP. Ainsi, lorsqu'une transformation allogène aboutit à la synthèse capsulaire normale, on trouve dans la bactérie un TP normal. Lorsque, au contraire, elle a pour résultat la restitution partielle de la synthèse capsulaire, on trouve dans la bactérie un TP nouveau, qui ne peut induire dans une bactérie « rough » que la formation d'une capsule réduite. Autrement dit, tout se passe comme si le TP de la bactérie mutée était plus ou moins « normalisé » sous l'action du TP, également muté, introduit dans le milieu. Quelle que soit la paire de TP donnant naissance à un TP normal, sa formation est un événement relative-

ment fréquent. La formation d'un TP partiellement restitué est, au contraire, un événement rare.

Les transformations allogènes ressemblent incontestablement aux recombinaisons décrites chez les bactériophages et le virus de l'influenza (2, 3, 5, 6, 8, 9). Ces recombinaisons ont lieu quand une cellule est infectée simultanément par deux virus différents, mais étroitement apparentés. En fait, le cas le plus étudié est celui d'une série de bactériophages mutés, dérivés de la lignée T2 (5, 6). Trois sortes de recombinaisons ont été reconnues:

a—Formation de particules de virus ayant des caractères spécifiques des deux souches infectantes (2, 3, 5, 6).

b—Formation de virus normal à partir de deux particules de virus inactivés par irradiation (8, 9).

c –Formation de virus normal à partir de paires de souches présentant le même caractère muté et devant leur origine à des mutations spontanées indépendantes au sein de la même souche. Il s'agit dans ce cas des mutations *r* (lyse rapide des bactéries infectées) du bactériophage T2. Quatorze mutants indépendants ont été étudiés. Une bactérie infectée par n'importe quelle paire de ces mutants peut donner un virus normal, mais la fréquence des recombinaisons dépend de la paire employée (5, 6).

Les recombinaisons de cette dernière sorte semblent présenter une analogie frappante avec les interactions entre TP au cours des transformations allogènes et il est tentant de chercher une base commune aux deux phénomènes. Dans ce qui suit, nous recommencerons par rappeler les hypothèses proposées pour l'explication des recombinaisons chez les virus; nous examinerons ensuite la possibilité d'appliquer ces hypothèses aux transformations allogènes.

Les hypothèses de Luria (8, 9) et de Hershey et Rotman (5, 6) ont en commun un postulat important, à savoir que le bactériophage est composé de plusieurs éléments génétiques distincts. Les auteurs cités admettent que les recombinaisons observées dans les bactéries infectées simultanément par plus d'une sorte de phage sont dues à des réassociations des éléments génétiques de deux particules de virus différents.

Les recombinaisons du type *a* sont expliquées par cette théorie sans autre hypothèse. Les recombinaisons du type *b* s'expliquent si l'on suppose que chacune des deux particules irradiées contient une mutation léthale différente. Enfin, les recombinaisons *c* s'expliquent si l'on admet que le phénotype *r* est dû, dans chacune des lignées examinées, à la mutation d'un locus différent.

Ces hypothèses ressemblent beaucoup à la théorie du crossing-over des chromosomes dont les auteurs se sont inspirés et sont plausibles surtout parce que le bactériophage est relativement grand et doué de propriétés biologiques variées: on connaît des mutations qui affectent la nature de la lyse, la capacité d'être adsorbé par un hôte donné, l'exigence des facteurs accessoires pour l'adsorption par la bactérie et la stabilité à la chaleur. Ainsi l'hypothèse selon laquelle les bactériophages sont composés de plusieurs gènes cadre bien avec les conceptions actuelles de l'étroite spécificité des gènes.

On peut se demander si une théorie analogue peut rendre compte des transformations allogènes. Ceci ne paraît pas facile pour les raisons suivantes:

1—Les TP capsulaires mutés du Type III n'ont apparemment qu'une seule activité phénotypique: l'induction de la synthèse plus ou moins active de polysaccharide capsulaire du type III. La spécificité de la catalyse de tous ces TP est la même et il n'y a donc pas de raison d'admettre que l'agent capsulaire est composé de plusieurs éléments génétiques distincts.

2—Pour rendre compte des données décrites dans ce travail par une théorie analogue à la théorie de crossing-over, il serait nécessaire de postuler au moins 6 gènes liés. Or, les propriétés chimiques et physicochimiques connues de l'agent capsulaire normal ne cadrent pas avec une telle idée. Comme nous l'avons indiqué plus haut, celui-ci semble être composé uniquement de l'ADN. Les études les plus récentes de l'histochimie des chromosomes montrent que ceux-ci doivent leur structure à une association des protéines et des acides ribonucléiques et désoxyribonucléiques. Aucune de ces trois classes de substances ne peut être considérée comme seule responsable de la structure linéaire. D'un autre côté, les extraits qui renferment l'agent capsulaire sont dépourvus de toute protéine décelable et ne renferment pas d'uracile, composé caractéristique de l'acide ribonucléique (7). De plus, la nature chimique de l'agent transformant capsulaire est indiquée clairement par son extrême sensibilité à l'action de la désoxyribonucléase cristallisée. La ribonucléase et la trypsine ne l'attaquent pas. L'agent capsulaire se déplace dans l'ultra-centrifuge et dans l'appareil de Tiselius avec les molécules d'acide désoxyribonucléique. Il n'y a aucune indication qu'il soit composé d'autres substances que l'acide désoxyribonucléique. Il est donc difficile de concilier les résultats de toutes les études chimique, physicochimique et biochimique de l'agent transformant avec l'idée qu'il est un fragment de chromosome. Il convient toutefois de remarquer que ces difficultés peuvent être dues au fait que nos connaissances chimiques de la structure linéaire des chromosomes sont fondées sur des études de la struc-

ture microscopique. A l'échelle macromoléculaire, nos connaissances des gènes sont nulles et c'est pourquoi les spéculations sur l'identité de l'agent capsulaire avec un ou plusieurs gènes ne peuvent pas être fructueuses.

On peut rendre compte des données relatives aux transformations allogènes en admettant que les agents mutés diffèrent entre eux par des régions altérées le long d'une structure linéaire. Il suffit de supposer:

1. Qu'une mutation du TP consiste en une altération localisée de sa structure.
2. Que l'agent capsulaire ait une polarité.
3. Que toute altération de sa structure ait pour effet une diminution de la sécrétion de polysaccharide capsulaire.
4. Que les différences dans la sécrétion du polysaccharide soient en rapport avec l'étendue de la région altérée.

On voit alors comment la combinaison de deux fragments d'agents capsulaires présentant des altérations dans des régions différentes peut permettre la reconstitution d'un TP normal; et comment les combinaisons de deux segments d'agents transformants à régions mutées qui se chevauchent ne peuvent conduire qu'à des reconstitutions partielles.

La figure 1 donne une représentation schématique de cette interprétation. La région altérée de l'agent SIII-1 est placée arbitrairement. Celle de l'agent SIII-1a est placée de façon à chevaucher la région mutée SIII-1, puisque l'interaction de ces deux agents ne donne que des reconstitutions partielles. Le TP SIII-1c est représenté avec une région mutée qui lui est particulière, parce qu'il donne des reconstitutions de l'agent normal avec les deux premiers. Enfin, l'agent SIII-2 est représenté avec une région mutée plus petite et en position différente des précédentes, parce qu'il donne des reconstitutions de l'agent normal avec n'importe lequel des autres agents mutés.

On notera que l'hypothèse présentée rend compte d'une caractéristique saillante du TP SIII-2a, celle notamment de ne pas donner de transformation allogène par action sur les souches SIII-1 et SIII-1a. Le TP SIII-2a possède, selon notre interprétation, la région mutée commune aux agents SIII-1 et SIII-1a, à l'interaction desquels il doit son origine. Il ne peut donc pas, en se recombinant avec un de ceux-ci, mener à la formation d'un TP qui ne présente pas cette région altérée. Notre hypothèse trouve en outre un appui important dans la rareté des transformations allogènes qui donnent naissance à des TP tels que SIII-2a. Ces transformations exigent en effet, selon notre schéma, des événements extrêmement précis, à savoir des recombinaisons qui éliminent les régions mutées particulières à chacun

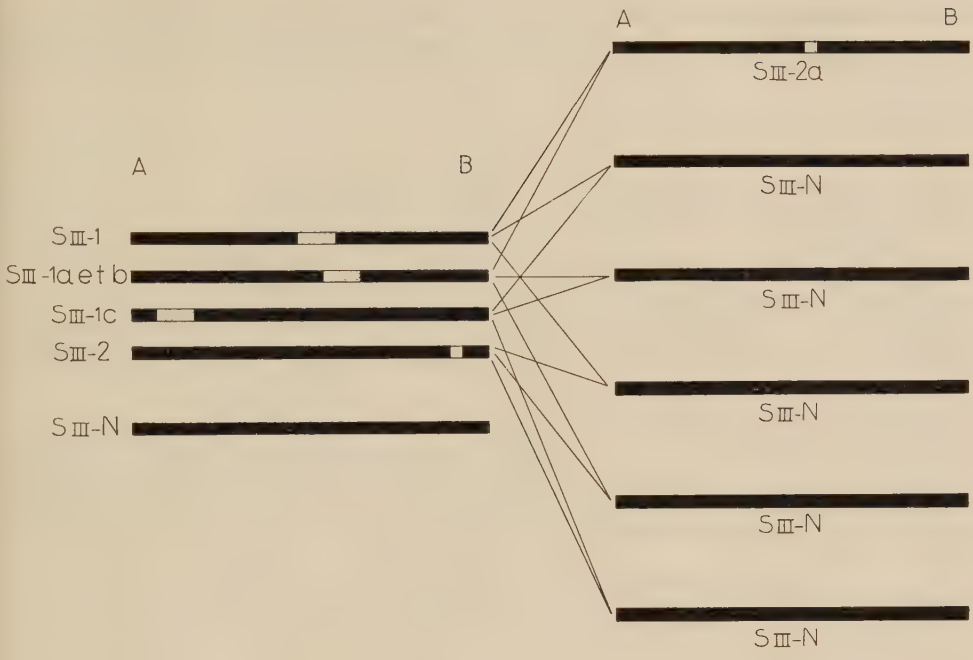


Fig. 1. Schéma des agents transformants mutés fondé sur les hypothèses décrites dans le texte. Les segments gris représentent les régions alterées par mutation spontanée. A gauche: agents transformants. A droite: restitution maxima à partir des fragments réunis de paires d'agents mutés.

des TP participants. Elles doivent donc être très rares et on a vu qu'elles le sont.

L'hypothèse formulée traduit en termes géométriques les données obtenues avec les divers agents capsulaires mutés. C'est peut-être un tort de construire une image géométrique, parce que, si l'agent capsulaire n'est qu'une molécule d'acide désoxyribonucléique, comme on peut le penser, il est peu probable qu'une simple image géométrique décrive l'état réel des choses. Mais, d'autre part, cette image suggère certaines expériences, telles que la recherche de pneumocoques contenant un agent à deux régions mutées, résultant aussi d'une transformation allogène et dont la démonstration serait un argument en faveur de l'interprétation proposée.

Toute spéculation mise à part, il est évident que l'agent capsulaire se comporte comme un élément composé de sub-unités. Faute de données plus précises sur la nature chimique et physicochimique de l'agent capsulaire,

et dans l'ignorance où nous sommes de ce qu'est l'autoreproduction d'une unité génétique, la définition de ces sub-unités est impossible à l'heure actuelle.

RÉSUMÉ

Les activités biologiques de cinq agents transformants capsulaires mutés du Type III ont été étudiées. Ces agents appartiennent à deux catégories: ceux (SIII-1) qui induisent la synthèse de l'antigène capsulaire du Type III en quantité insuffisante à la formation d'une capsule complète, et ceux (SIII-2) qui induisent la formation d'une capsule du Type III complète, mais encore de dimensions réduites par comparaison avec la capsule de pneumocoques normaux.

Chacun de ces agents isolés sous forme d'acide désoxyribonucléique induit, dans des bactéries sans capsule, la formation d'une capsule identique à celle du pneumocoque dont l'agent a été extrait. Ces inductions, tout à fait analogues aux transformations classiques, ont été appelées *transformations autogènes.*

On peut confronter deux agents mutés dans une seule bactérie, puisque la transformation des pneumocoques mutés de la catégorie SIII-1, qui secrètent très peu de polysaccharide, est encore possible. Dans ce cas, on observe généralement l'apparition de pneumocoques dont la synthèse capsulaire est à la fois plus active que celle de la souche traitée et celle de la souche qui a fourni l'agent transformant. Ces inductions ont été appelées *transformations allogènes.*

Le degré d'augmentation de la synthèse capsulaire, qui résulte d'une transformation allogène, dépend de la paire d'agents confrontés.

Les diverses transformations allogènes n'ont pas lieu avec la même facilité, certaines d'entre elles étant très rares.

Les transformations allogènes sont dues à la formation d'agents transformants dont l'activité, au point de vue de la synthèse capsulaire, est accrue. Cette activité atteint, dans certains cas, celle des agents normaux. L'étude des transformations allogènes montre que les mutations spontanées et indépendantes de l'agent capsulaire normal en agent SIII-1 ne sont pas nécessairement identiques. Parmi les quatre agents de cette catégorie étudiés, trois sont différents.

Au sein d'une population, un même agent muté peut induire simultanément des transformations autogènes et allogènes.

Un nouvel agent muté, résultat d'une transformation allogène, a été identifié. Cet agent, qui confère aux pneumocoques un phénotype sem-

blable au phénotype appelé SIII-2, présente des activités transformantes qui lui sont particulières et représente donc un nouvel agent qui n'a jamais été trouvé dans la nature.

La structure possible de l'agent capsulaire est discutée à la lumière des résultats observés.

LITTÉRATURE CITÉE

1. Avery O. T., MacLeod, C. M., et McCarty, M., *J. Exptl. Med.*, **79**, 131 (1944).
2. Burnet, F. M., *Trans. N. Y. Acad. Sci.*, **13**, 2 (1950).
3. Delbrück, M., et Bailey, W. T., Jr., *Cold Spring Harbor Symposia Quant. Biol.*, **11**, 33 (1946).
4. Griffith, F., *J. Hyg.*, **27**, 113 (1928).
5. Hershey, A. D., et Rotman, R., *Proc. Natl. Acad. Sci.*, **34**, 89 (1948).
6. —— *Genetics*, **34**, 44 (1949).
7. Hotchkiss, R. D., *Colloques Intern. du C. N. R. S.*, **VIII**, 57 (1948).
8. Luria, S. E., *Proc. Natl. Acad. Sci.*, **33**, 253 (1947).
9. —— *Genetics*, **34**, 92 (1949).
10. MacLeod, C. M., et Krauss, Marjorie, *J. Exptl. Med.*, **86**, 439 (1947).
11. McCarty, M., et Avery, O. T., *J. Exptl. Med.*, **83**, 97 (1946).
12. McCarty, M., Taylor, H. E., et Avery, O. T., *Cold Spring Harbor Symposia Quant. Biol.*, **11**, 177 (1946).
13. Sevag, M. G., *Biochem. Z.*, **273**, 419 (1934).
14. Taylor, H. E., *Colloques Intern. du C. N. R. S.*, **VIII**, 45 (1948).
15. —— *J. Exptl. Med.*, **89**, 399 (1949).

THE ORGANIZER AND RESPIRATION IN *RANA PIPIENS*

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The relationship between the organizer and respiration in the amphibian embryo involves two problems. First is the question of whether or not the organizer owes its inductive power to its intense respiration, as postulated by Child. With respect to this problem, a great deal of work has been done in the past twenty years. This work includes the study of the respiratory activity of the organizer in contrast to that of the other parts of the gastrula, and the study of the relationship between respiratory inhibitors and activators and inductive power. The general impression is that the organizer does not owe its inductive power to its intense respiration.

The second problem is whether or not neural induction is associated with an increase in respiration. This question, strangely enough, has been almost neglected by investigators. The purpose of the present work is to deal with this problem.

METHODS

A. Method of dissection. The respiration of different eggs, as well as of different parts of the same embryo, varies. Unless a large number of determinations are made, which is almost impractical, a more careful design of experiments is needed in order to obtain valid information. Such variations can be eliminated to a great extent by using the lateral halves of the same parts of the same embryos, assuming that there is no respiratory asymmetry from the left side to the right side of the embryo. In order to determine whether such respiratory asymmetry exists several experiments were made in advance, using the left and right parts of the embryo. The regions used are shown in figure 1. The reason for using these widely separated regions instead of adjacent ones is that any differences that may exist between right and left regions should be more pronounced. The data presented in table I indicate that a respiratory asymmetry from left to right sides of the gastrula may exist (fig. 2). On the basis of dry weight, the respiratory activity of the left part is probably higher. Boell (1), on the basis of total nitrogen, found the same phenomenon. In using the dissection method, therefore, one must take this fact into consideration.

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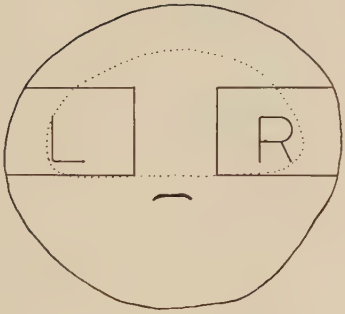

Fig. 1.

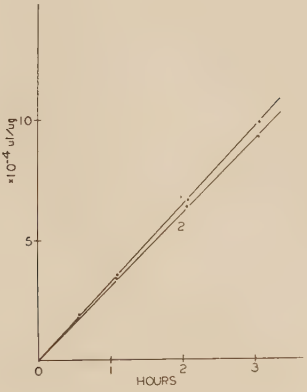

Fig. 2.

Fig. 1. Diagram showing method of dissection for right and left fragments of gastrula.
Fig. 2. Respiration of the left (1) and right (2) pieces. Dissection as in Fig. 1.

TABLE I

Egg No.	Rate of left piece $\mu l \times 10^{-4}/\mu g/hr$	Total Dry Wt. μg	Rate of right piece $\mu l \times 10^{-4}/\mu g/hr$	Total Dry Wt. μg
1	3.1	56	2.3	56
2	3.4	50	1.7	57
3	3.1	40	3.0	40
4	2.2	44	2.5	42
Mean	3.0		2.5	

The essential experimental procedure used in the present studies is shown in fig. 3. In fig. 3 A, the presumptive neural plate and a part of the presumptive epidermis were used as the host for the organizer region, while in fig. 3 B presumptive epidermis was used. In both cases the "two-in-two" (organizer and host separated) and the "two-in-one" (organizer and host united) were prepared alternatively from the left and right lateral halves. For instance, when one set of determinations was made on the left half or halves in the form of the "two-in-one" and the right half or halves in the form of the "two-in-two," the next set of determinations employed the left half or halves in the form of the "two-in-two" and the right half or halves in the "two-in-one" form. With this procedure it is apparent that the "two-in-one" relationship will, in most cases, involve neural induction, while the "two-in-two" will not. Respiration of the "two-in-one" and the "two-in-two" regions from the same egg was then compared.

All eggs used for the dissections were either at stage 10 or at stage 10 $^1/_2$ (7).

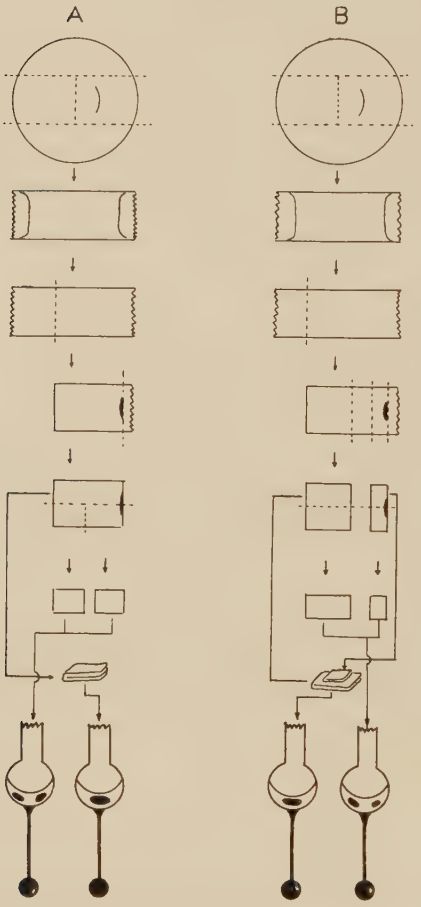

Fig. 3. Method of dissection A. The region containing the organizer and the presumptive neural plate is removed from the gastrula. It is then bisected. One fragment is folded to bring the organizer in *contact* with the presumptive neural plate. The other fragment is cut in half to *separate* the organizer from the presumptive neural plate. B. Similar to A but in one case the organizer is *combined* with the presumptive *epidermis* while in the other it is separated from the presumptive epidermis.

B. Method of cultivation. The eggs were dissected in Holtfreter's solution without carbonate. In some experiments the explants were kept in this fluid throughout the cultivation period after operation. In other experiments they were transferred into $^1/_2$ Holtfreter's solution without carbonate after healing, and were then returned to the modified Holtfreter's solution just before measurement of their respiration. All explants were cultured at 12° C in small finger bowls until the desired stage, neural fold. In each set of experiments some intact embryos were kept side by side with the explants to indicate the stage of development.

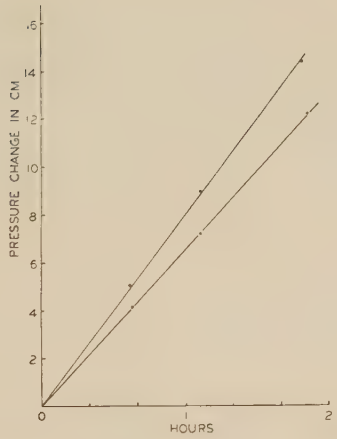

Fig. 4. Pressure changes in divers containing the "two-in-two" explants (open circles) as compared with the "two-in-one" explants (solid circles).

C. Methods of measuring oxygen uptake and dry weight. The measurements of oxygen uptake were made by means of a Cartesian diver respirometer (for detail, see Sze (8)). All explants were measured in Holtfreter's solution without carbonate at 25° C. When the explants in the form of the "two-in-two" were measured, the two pieces were put into the same diver (see fig. 3). After the respiratory determinations had been made, the explants were washed with distilled water and dried in a hot oven over night. The dry weights were then determined on a quartz fiber balance.

A general idea of the total pressure changes at two hours can be obtained from fig. 4.

RESULTS

In total, nine sets of experiments were made, the data of which are given in table 2. Six sets were made with organizer and the presumptive neural plate and three sets, on the organizer and the presumptive epidermis. Of the six sets on the organizer and neural plate, four showed that the "two-in-one" combination has higher respiratory activity, while only two showed that the respiratory activity of the "two-in-two" is higher than that of the "two-in-one." One of these two cases, moreover, shows a respiratory rate abnormally low for this stage of development. This measurement, accordingly, was not included in our calculation of the means. All three sets of the organizer and the presumptive epidermis showed invariably that the "two-in-one" combination has a higher respiratory rate. As a whole, there is a

TABLE II

Egg No.	Rate of the "two-in-two" explant $\mu l \times 10^{-4}/\mu g/hr$	Total Dry Wt. μg	Rate of the "two-in-one" explant $\mu l \times 10^{-4}/\mu g/hr$	Total Dry Wt. μg	Stage of development	Host tissue	Culture medium
1	6.0	104	6.9	107	15	P.N.P.	$^1/_2$ Holtfreter minus HCO
2	5.2	174	5.0	162	»	»	»
3	5.2	110	5.5	113	»	»	»
4	3.8	90.4	6.1	88	»	»	»
5	2.9[1]	67.4	2.7[1]	89	»	»	Holtfreter minus HCO
6	4.6	107	5.2	96.3	»	»	»
7	5.6	104	6.2	123	14	P.E.	»
8	5.2	119	6.0	102	»	»	»
9	5.2	73.5	6.3	63.5	»	»	»
Mean	5.1		5.9				

P.N.P. presumptive neural plate.
P.E. presumptive epidermis.
The difference between the means is significant by using individual comparison, $P = 0.02 - 0.01$.

statistically significant difference between the "two-in-one" and the "two-in-two" explants. The former show about 16 per cent higher respiratory activity.

DISCUSSION

The difference found between the "two-in-one" and the "two-in-two" explants cannot be due to technical factors, since care was taken to eliminate several sources of error.

1) The possible difference between the lateral halves of the same parts of the gastrula has been eliminated by using the left and right halves alternatively from egg to egg. The observed difference, therefore, cannot be ascribed to bilateral asymmetry.

2) In the experiments with the organizer and the presumptive neural plate the "two-in-two" explants were subjected to one more cut than were the same regions prepared as "two-in-one." This extra cut might injure the tissue and, consequently, the "two-in-two" explants would respire at a different rate. However, in the case of the organizer and the presumptive epidermis, where the number of cuts is the same for both the "two-in-one"

[1] Not included in the calculation of the means.

and the "two-in-two" preparations, the results are the same as for organizer plus presumptive neural plate.

3) The surface-volume ratios of the "two-in-two" and the "two-in-one" preparations are different. This kind of difference, however, would not yield the present results. Larger surface-volume ratios should favor gaseous exchange, and consequently favor respiration. Although the "two-in-two" explant has a larger ratio of surface to volume, it nevertheless shows a lower rate of respiration.

From the above considerations it is concluded that the difference in respiratory activity must be real and not an artefact. The question then arises as to which of the two parts of the "two-in-one" explant is responsible for the increase of respiration—the ectoderm or the organizer, or both? The present study, unfortunately, cannot answer this question.

What use is made of the energy released from the increased respiration in the "two-in-one" explants? The possibility that the energy is used for cellular movements seems unlikely, since there can be relatively little cell migration in explants. Jaeger (4) has shown that while glycogen decreases during invagination of the dorsal lip region in intact gastrulae, explants of the same region show no such decrease.

Since the experiments of Holtfreter (3) and Schechtman (6) it has been known that the ectoderm, when isolated and cultivated *in vitro*, differentiates into epidermis only. The organizer region, however, will self-differentiate in explants, usually giving rise to notochord and mesoderm tissues in the case of a small piece of organizer (Laposchov, cited by Needham, (5)). We know that the organizer will induce neural differentiation of the ectoderm when they are in contact. On the other hand, we have no idea whether or not in this system the ectoderm has any effect on the differentiation of the organizer. However, we do know that the ectoderm has potency to become mesenchyme and muscle (2). Thus, the difference between the "two-in-one" and the "two-in-two" combinations lies in the fact that the former produces neural differentiation, which the latter more likely does not. Probably, the energy from the increased respiration of the "two-in-one" explants is used for neural differentiation.

SUMMARY

It has been found that the respiratory activity of the combined piece of organizer and ectoderm is higher than that of the separated pieces of these two regions, when they are isolated at gastrula stage and measured at neurula stage.

REFERENCES

1. Boell, E. J., *Ann. N. Y. Acad. Sci.*, **49**, 773 (1948).
2. Holtfreter, J., *Arch. Entwicklungsmech.*, **138**, 163 (1938).
3. —— *Ibid.*, **138**, 657 (1938).
4. Jaeger, L., *J. Cellular Comp. Physiol.*, **25**, 97 (1945).
5. Needham, J., Biochemistry and Morphogenesis. Cambridge, University Press, 1942.
6. Schechtman, A. M., *Univ. Calif. Pubs. Zool.*, **39**, 393 (1935).
7. Shumway, W., *Anat. Rec.*, **78**, 139 (1940).
8. Sze, L. C. (in preparation).

ANTIGENIC TRANSFORMATIONS IN *PARAMECIUM AURELIA*, VARIETY 4, STOCK 51 UNDER THE INFLUENCE OF TRYPSIN AND CHYMOTRYPSIN[1]

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Eight antigenically diverse strains have arisen within stock 51 of variety 4 of *Paramecium aurelia* (3). Each of these eight types is inherited under standard conditions of culture (27° with enough food to permit one fission per day), maintaining itself through both vegetative and sexual reproduction. Exposure to immobilizing antisera can transform every one of the types to other types falling within the spectrum of the types characteristic of the stock. These transformations are hereditary. Sonneborn has interpreted the interconvertibility of the types within a stock to indicate that each hereditary type possesses a primary antigen (the one involved in the immobilization by antiserum) and a number of secondary antigens, either actually present in small amounts, or always potentially producible. Only those antigens belonging to the spectrum of the stock can function as primary antigens. Other environmental conditions, such as temperature and food supply can also cause a transformation of antigenic type.

It would seem probable that a transformation induced by antiserum is the result of a suppression or a retardation of the multiplication of the antigen with which the antibody in the serum combines. Then one of the other antigens, whichever is best adapted to the new prevailing conditions, replaces the suppressed one. Removal of the primary antigen, or decreasing its concentration might then also induce a transformation. Lancefield (2) reported that the M-antigens could be removed from Streptococci by exposure to trypsin. Kimball (1) found that exposure of stock 60 of variety 1 of *Paramecium aurelia* resulted in the production of a high proportion of animals resistant to the action of the specific antiserum. These results

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were interpreted to mean that the resistance is produced by the loss or the inactivation of the antigens by trypsin.

Exposure of *Paramecium aurelia*, variety 4, stock 51 to the action of trypsin and chymotrypsin might therefore well induce transformation to other types within the spectrum of the stock.

METHODS

1. Stocks. Six different antigenic types of *P. aurelia*, variety 4, stock 51 (types 51 A, 51 B, 51 C, 51 D, 51 E, 51 G) were kept in antigenically pure culture under standard conditions of growth (27°, with enough food added daily to permit one fission per day).

2. Enzyme preparations. The enzymes used were commercially available crystalline preparations (Armour and Company). Since the trypsin and chymotrypsin preparations contained considerable amounts of salt (ammonium sulfate and magnesium sulfate, respectively) the enzymes were dissolved in distilled water and dialyzed against running cold distilled water for 24 hours. The dialyzates were dried in the frozen state and thawed immediately before use.

3. Concentration of the enzymes. The sensitivity of *P. aurelia* towards trypsin and chymotrypsin was determined by incubating the animals in graded amounts of these enzymes. It was found that the maximal final concentration to permit survival for 24 hours of at least 5 per cent of the original number of animals was 5 mg/ml. The corresponding maximal final concentration of chymotrypsin was found to be 0.5 mg/ml.

4. Incubation with trypsin. To a test tube were added 1 ml of phosphate buffer (pH 7.2), enough of the enzyme solution to make a final concentration of 5 mg/ml, and so much of a *P. aurelia* culture (in lettuce infusion) to make the final volume 10 ml. A second tube, serving as the control, was prepared similarly except that the enzymes solution was replaced by an equal amount of distilled water. The tubes were placed in a constant temperature water bath at 37°. The initial concentration of *P. aurelia* present per ml of the final solution was determined for both tubes. The animals were tested for antigen types. After 12 and 24 hours of incubation counts were made of the animals present per ml in each tube and the antigenic type or types present in each culture were determined.

5. Incubation with chymotrypsin. The procedure followed was identical with that described for trypsin, with two exceptions. A phosphate buffer of pH 8 was used and the final concentration of chymotrypsin was 0.5 mg/ml.

6. Single isolations. After 12 and 24 hours of incubation one or two ml of fluid containing *P. aurelia* were removed from the tubes. The animals were transferred with a micropipette to culture fluid (a regular 24 hour-old culture of Aerobacter aerogenes in lettuce infusion, diluted 1 : 10 with Ringer's solution before use). Three sets of 100 single isolations from each tube were incubated at 20°, 27°, and 32°. After the animals under each temperature condition had undergone approximately 8 fissions, the resulting clones were tested for antigenic types.

7. Mass cultures. *P. aurelia* of antigenic type 51 A, 51 B, 51 C, and 51 D were

treated with trypsin and chymotrypsin in the same manner as described under 4 and 5. After 12 and 24 hours of incubation animals were removed from the tubes containing the enzyme solutions and from the control tubes. After these animals had been washed once with regular culture fluid 300 ciliates were distributed over three tubes containing culture fluid (approximately 100 animals per tube). These tubes were incubated respectively at 20°, 27°, and 32°. After the animals in each tube had gone through approximately 8 fissions the cultures were tested for antigenic type.

RESULTS

It is clear from table I that exposure to the proteolytic enzymes has a general injurious effect on *P. aurelia*. Animals of types 51A, 51D and 51G are least affected by the primary exposure to trypsin. Types 51B and 51E are very susceptible to the action of the enzymes. A large percentage of the visibly unaffected animals of all types dies after they have been removed from the enzyme solutions, indicating that the injurious consequences of exposure to the enzymes extend even to those that seem unaffected at the time of removal from the enzyme solutions. This delayed effect is, on the whole, strongest in type 51G. The total lethal effect is greatest for type 51B and type 51E after a 12 hours exposure to trypsin, while it is lower and practically the same for types 51A and 51C. It is somewhat less for type 51G. Type 51D seems to be the most resistant to the actions of trypsin the total lethal effect of a 24 hours exposure to trypsin is practically the same for types 51B, 51E and 51G. It might be slightly less in the case of type 51A and type 51C. Type 51D is again the most resistant to the action of trypsin. Of all types, type 51B is most susceptible to the action of chymotrypsin, while the other types are all affected to a much lesser degree. There seems to be no significant difference in susceptibility to chymotrypsin among these other types.

The change in antigenic type immediately after exposure is given in table II. No change in antigenic type was detected in type 51A and type 51B animals by exposure to either trypsin or chymotrypsin. Some animals of type 51C had transformed to types 51A and 51B after both 12 and 24 hours exposure to trypsin, resulting in the presence of animals of types 51A, 51B and 51C in the cultures. Exposure to chymotrypsin for 12 hours resulted in a disappearance of the 51C antigen which was replaced by the 51B antigen. After 24 hours type 51A was also present in the cultures. Exposure of animals of type 51D to trypsin for 12 hours caused the disappearance of the 51D antigen in part of the animals, while exposure for 24 hours to trypsin resulted in the loss of the 51D antigen in all surviving animals.

TABLE I

Lethal Effect of Trypsin and Chymotrypsin on Antigenic Types of *P. aurelia*.

Type	Temperature of single isolations	Trypsin						Chymotrypsin					
		12 hours			24 hours			12 hours			24 hours		
		L.E.[1] during exposure	L.E. after exposure in single isolations	Total L.E.	L.E. during exposure	L.E. after exposure in single isolations	Total L.E.	L.E. during exposure	L.E. after exposure in single isolations	Total L.E.	L.E. during exposure	L.E. after exposure in single isolations	Total L.E.
A		59			76			48			48		
	20		29	71		69	93		5	51		17	57
	27		23	68		69	93		7	52		4	60
	32		49	79		59	90		10	58		29	73
B		93			93			85			94		
	20		48	96		39	96		90	98		81	99
	27		29	95		31	96		95	99		77	99
	32		50	96		47	96		94	99		84	99
C		72			89			66			57		
	20		7	74		37	93		2	67		6	60
	27		5	78		31	92		8	69		5	60
	32		16	76		52	95		13	71		11	62
D		34			74			55			57		
	20		11	42		37	84		8	59		8	60
	27		11	42		12	77		11	60		4	59
	32		4	37		39	84		9	60		18	65
E		92			95			47			70		
	20		20	94		29	96		9	52		9	63
	27		20	94		13	96		2	48		14	64
	32		21	94		23	96		9	52		14	64
G		25			65								
	20		63	72		89	96						
	27		47	60		95	98	—	—	—	—	—	—
	32		38	53		97	99						

TABLE II

Antigenic type immediately after exposure to trypsin and chymotrypsin for 12 and 24 hours.

Type	Control		Trypsin		Control		Chymotrypsin	
	12 hours	24 hours	12 hours	24 hours	12 hours	24 hours	12 hours	24 hours
A	A	A	A	A	A	A	A	A
B	B	B	B	B	B	B	B	B
C	C	C	A, B, C[1]	A, B, C[1]	C	C	B	A, B[1]
D	D	D	D, O[2]	O[2]	D	D	O[2]	O[2]
E	E	E	E	E	E	E	O[2]	O[2]
G	G	G	G	G	—	—	—	—

A similar result was obtained by exposure of 51D type to chymotrypsin for either 12 or 24 hours. In all of these cases, the animals which failed to react to anti-D sera also failed to react to all the other antisera available at this time (anti-A, B, C, E, G and H). Only one other antigenic type has subsequently been identified in the spectrum of possibilities of stock 51, namely type 51J. Type 51D is known to transform to type 51J under certain conditions. The type J is highly unstable (Sonneborn and Whallon, personal communication). The non-reactive animals obtained in this experiment persisted in this condition for at least 20 fissions, but they could not be maintained pure at 27°; they reverted to type 51D or changed to types 51A or 51G. In view of the considerable interest of such regularly directed transformations the experiment was repeated on a small scale with type 51D alone. Exposure to trypsin for 12 hours resulted in a mixture of 51D and 51J animals, while exposure to chymotrypsin for the same length of time gave rise to only type 51J animals. Exposure to either enzyme for 24 hours resulted in the appearance of only type 51J animals.

Exposure of type 51E to chymotrypsin for 12 hours did not cause a detectable change in antigenic type. Exposure to chymotrypsin for 24 hours resulted, as in the case of 51D animals, in the appearance of non-reactive animals. These might have belonged to type 51J. As in the case of 51D animals, efforts to obtain pure cultures of the non-reactive type failed. The type transformed to type 51A or type 51G.

Figures 1–6 give the amount and direction of transformation in series of cultures derived from single isolations of animals that had been exposed

[1] Mixtures of 51 A animals with 51 B and 51 C animals.

[2] O denotes that the *P. aurelia* were resistant to the antisera available at the time of the experiment.

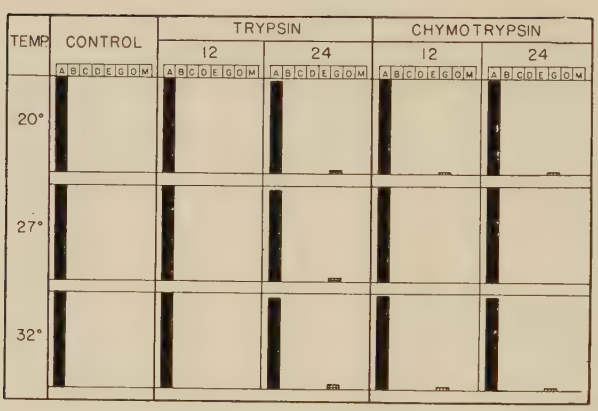

Fig. 1. Transformation of type 51 A after exposure to trypsin and chymotrypsin for 12 and 24 hours, followed by growth in single isolations for approximately 8 fissions at 20°, 27° and 32°. The length of each bar represents the percentage of the surviving clones that have transformed to the type indicated by the shading.

to the two proteolytic enzymes, then removed and allowed to go through approximately 8 fissions at 20°, 27°, and 32°, respectively, before being tested for antigenic type.

Antigen type 51A seems to be the most stable type. Under the conditions of the experiment the controls of this type were stable at the three temperatures. Some changes to antigenic type 51G could be detected in the surviving clones at 20° after pre-treatment with trypsin for 24 hours and after pre-treatment with chymotrypsin for 12 and 24 hours. At 27° about 5 per cent of the surviving clones after a 24 hours exposure to trypsin had transformed to type 51G. No changes in antigenic type could be detected in clones grown at 32°.

A small amount of transformation to type 51G (about 1 per cent) was observed in the controls of 51B when grown at 20°. A slightly larger percentage transformed when the animals had been exposed to trypsin for 12 hours. The trypsin effect was more pronounced after 24 hours. The mixed clones consisted of animals of type 51B and 51G. No effect on antigenic type was detectable after the exposure to chymotrypsin. The antigenic type of animals of type 51B was stable when the controls were grown at 27°. Pre-treatment with trypsin for 12 hours and 24 hours gave rise to some transformations. One per cent of the animals had transformed to type 51G. The mixed clones consisted of animals of type 51A and type 51B. The pre-exposure to chymotrypsin gave rise to one clone that consisted of animals of type 51A and type 51B. It has to be taken into account that the survival

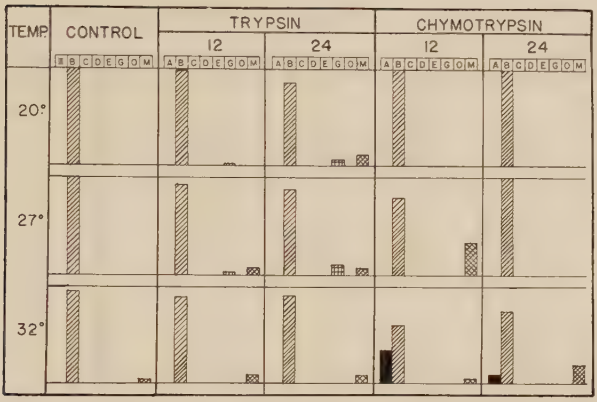

Fig. 2. Transformation of type 51 B after exposure to trypsin and chymotrypsin for 12 and 24 hours, followed by growth in single isolations for approximately 8 fissions at 20°, 27°, and 32°. The length of each bar represents the percentage of the surviving clones that have transformed to the type indicated by the shading.

rate in the case of type 51B is extremely low; consequently, the amount of observed transformation with animals of type 51B could be considered as a minimal figure. Some transformation to type 51G took place when the animals were pre-treated with trypsin for 24 hours. One clone, consisting of animals of type 51A and 51B was also found. The controls of type 51B, when grown at 32° showed a tendency to transform to type 51A. A small number of mixed clones, consisting of animals of type 51A and animals of type B could be detected after growth at that temperature. Exposure to trypsin, followed by growth at 32° resulted in the appearance of some mixed clones of type 51A and 51B after a 12 hour exposure and the formation of a small amount of type 51A after 24 hours exposure to trypsin. Exposure to chymotrypsin resulted in an apparently greater transformation to type 51A than did exposure to trypsin. A discrepancy is indicated by the fact that exposure for 12 hours to chymotrypsin resulted in more transformation to type 51A than an exposure for 24 hours. However, the low survival rate of the 51B animals would tend to invalidate the significance of this discrepancy, since the sampling of such small numbers would not be reliable.

Antigen type 51C is the most unstable type under the conditions of these experiments. At 20° the controls showed a great deal of transformation, mostly to type 51A (21 per cent). The mixed clones at this temperature consisted of animals of type 51A and type 51C (14 per cent), and one mixed clone of types 51A, 51C and 51G. Exposure to trypsin for 12 hours followed

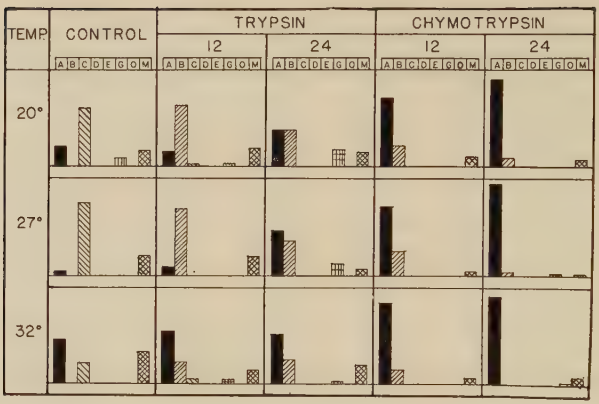

Fig. 3. Transformation of type 51 C after exposure to trypsin and chymotrypsin for 12 and 24 hours, followed by growth in single isolations for approximately 8 fissions at 20°, 27° and 32°. The length of each bar represents the percentage of the surviving clones that have transformed to the type indicated by the shading.

by growth for approximately eight fissions at 20° resulted in the almost complete elimination of type 51C. Transformation was mainly to type 51B while the transformation to type 51A was somewhat lower than in the control (14 per cent as compared to 21 per cent). The mixed clones consisted of types 51A and 51B (5 per cent), 51A and 51G (3 per cent), 51B and 51G (5 per cent), and 51A, 51B and 51G (5 per cent). Exposure to trypsin for 24 hours resulted in an even distribution of clones of type 51A and type 51B, a larger number of clones transformed to type 51G and mixed clones consisting of types 51A and 51B (7 per cent), 51B and 51G (3 per cent), 51A and 51G (2 per cent). Exposure to chymotrypsin resulted in the transformation of a large percentage of the surviving clones to type 51A, both after 12 hours and after 24 hours. The mixed clones consisted in each case of animals of type 51A and animals of type 51B. Type 51C is more stable at 27°. Even at this temperature transformation to type 51A takes place. The mixed clones (21 per cent) consisted of animals of type 51A and type 51C. Treatment with either trypsin or chymotrypsin increases the tendency to transform to type 51A, chymotrypsin being more effective in this respect. Pre-treatment with trypsin for 24 hours, followed by growth at 27° resulted in some transformation to type 51G (10 per cent). The mixed clones consisted of animals of type 51A and type 51B (2 per cent), type 51B and 51G (1 per cent), and 51A and 51G (1 per cent). Type 51C is also unstable at 32°. Transformation is to type 51A (45 per cent). The mixed clones consisted mainly of mixtures of animals of type 51A and type 51C (27 per cent).

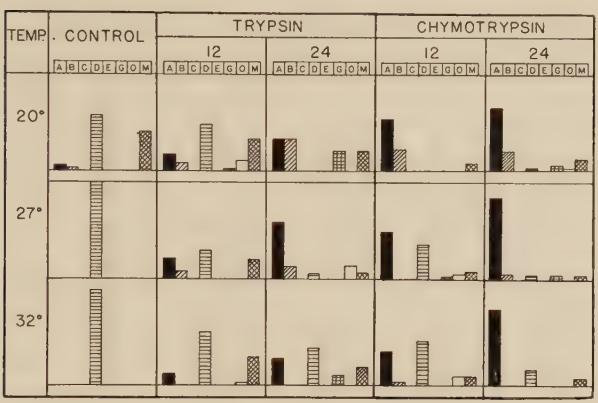

Fig. 4. Transformation of type 51 D after exposure to trypsin and chymotrypsin for 12 and 24 hours, followed by growth in single isolations for approximately 8 fissions at 20°, 27° and 32°. The length of each bar represents the percentage of the surviving clones that have transformed to the type indicated by the shading.

Some mixed clones of type 51B and 51C (5 per cent) were also present. Treatment with trypsin and chymotrypsin results in a greater transformation to type 51A. Part of this is undoubtedly due to the temperature effect which tends to exaggerate the transformation to type 51A. In every instance, however, exposure to chymotrypsin results in a larger number of clones that have transformed to type 51A, than does exposure to trypsin. Type 51B does not appear as a result of the temperature effect. The controls do not result in transformation to this type.

Antigen type 51D is unstable at 20°. Preferential transformation to type 51A is indicated here, by the fact that 5 per cent of the clones have already transformed to type 51A, and that the mixed clones consist of mixtures of different types: type 51A and 51D (25 per cent), type 51A, 51B and 51D (10 per cent), type 51A and 51B (1 per cent). Some transformation to type 51B also takes place at this temperature (1 per cent). Of the mixed clones 5 per cent are mixtures of animals of type 51B and 51D. Exposure to the two proteolytic enzymes induces more transformation than the exposure to the temperature of 20° only. Type 51G appears when the animals have been treated with trypsin for 12 hours. This appears more frequently when the trypsin treatment has lasted for 24 hours. Pretreatment with chymotrypsin seems to result in the appearance of more clones of type 51A than does pretreatment with trypsin. While type 51D is stable at 27°, treatment with either trypsin or chymotrypsin induces a large amount of transformation. Again, treatment with chymotrypsin induces more transformation to type

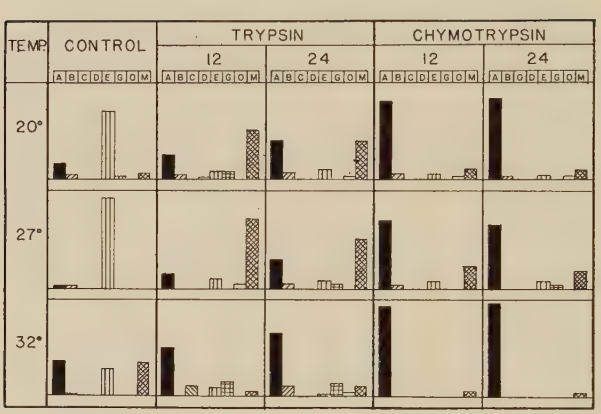

Fig. 5. Transformation of type 51 E after exposure to trypsin and chymotrypsin for 12 and 24 hours, followed by growth in single isolations for approximately 8 fissions at 20°, 27° and 32°. The length of each bar represents the percentage of the surviving clones that have transformed to the type indicated by the shading.

51A than does treatment with trypsin. Those types that did not react to any antisera were more stable at this temperature and showed up more frequently in the clones grown up from the single isolations. This is particularly true of the clones resulting from the pre-treatment with trypsin. Only a very small amount of non-reactive clones were evident after exposure to chymotrypsin for 12 hours, and none after exposure to this enzyme for 24 hours. Type 51B showed up in small numbers after trypsin treatment for 12 and 24 hours, and after chymotrypsin treatment for 24 hours. Type 51D is also stable at 32° (under the conditions of the experiment), in contrast to the high degree of transformation obtained after exposure to trypsin and chymotrypsin. Exposure to chymotrypsin results in a greater degree of transformation to type 51A. There is however a difference in response to the enzyme treatment between the single isolations grown at 32° and those grown at 27°. More type 51D appears in the clones when grown at 32°.

Type 51E resembles type 51C in the pattern of transformation. At 20° this type transforms mostly to type 51A, and in lesser degree to types 51B and 51G. Exposure to trypsin for 12 hours causes more transformation to type A than found in the control. Some clones of type 51B (3 per cent), type 51D (1 per cent), and type 51G (7 per cent) are formed. The large number of mixed clones consists of the following mixtures: types 51A and 51E (8 per cent), 51E and 51G (13 per cent), 51G and non-reactive animals (3 per cent), 51A and non-reactive animals (4 per cent), 51B and 51E (5 per cent), 51A, 51G and 51E (2 per cent), 51E, 51G and non-reactive animals

(1 per cent), 51B and 51G (1 per cent), 51A and 51G (1 per cent), 51A and 51B (1 per cent), and 51B, 51E and 51G (2 per cent). Apparently the pattern of transformation was not yet set after 8 fissions. A similar, but slightly less complex pattern was evident after 24 hours exposure to trypsin. More animals had transformed to type 51A, and types 51D and 51G had disappeared. The mixed clones still showed a large variety of types; 51A and 51B (1 per cent), 51A and 51E (9 per cent), 51A and 51C (1 per cent), 51E and 51G (7 per cent), 51B and 51E (2 per cent), 51B and 51G (3 per cent), 51A and non-reactive animals (2 per cent), 51E and non-reactive animals (2 per cent), 51B and non-reactive animals (2 per cent) and 51A, 51B and non-reactive animals (1 per cent). Treatment with chymotrypsin resulted in the formation of a large number of type 51A clones. The mixed clones were still very complex, those after a 12 hours exposure consisting of the following: 51A and 51E (2 per cent), 51B and 51E (2 per cent), 51A and 51B (2 per cent), 51A and non-reactive animals (1 per cent), 51E and non-reactive animals (1 per cent), 51A, 51E and 51G (1 per cent), and 51B, 51E and non-reactive animals (1 per cent). The mixed clones present after 24 hours treatment with chymotrypsin consisted of types 51A and 51E (10 per cent), and 51A and non-reactive animals (1 per cent). Type 51E is fairly stable at 27°, only a small number transforming to types 51A and 51B. Exposure to trypsin and chymotrypsin again results in a large amount of transformations. Type 51A becomes more prominent after treatment with the two enzymes. Treatment with chymotrypsin results here also in more transformation to type 51A than does exposure to trypsin. The treatment with trypsin again gives rise to a large number of mixed clones. After a 12 hour exposure to trypsin followed by growth at 27° the following mixed clones were found: type 51A and 51E (27 per cent), 51A and 51B (2 per cent), 51A and 51G (4 per cent), 51B and 51E (3 per cent), 51E and 51 G (9 per cent), 51A, 51E, and 51G (5 per cent), 51B, 51E and 51G (1 per cent), 51E and non-reactive animals (3 per cent), 51B, 51E, and non-reactive animals (3 per cent), 51A, 51B and 51E (2 per cent). Exposure to trypsin for 24 hours gave rise to more clones of type 51A, the appearance of a few clones of type 51B (5 per cent) and a large number of mixed clones, 51A and 51E (8 per cent), 51B and 51E (9 per cent), 51E and 51G (14 per cent), 51E and non-reactive animals (4 per cent), 51A and non-reactive animals (2 per cent), and 51B, 51E and 51G (1 per cent). The mixed clones resulting from the chymotrypsin treatment were less complex. Those appearing after a 12 hour treatment consisted of the following mixtures: 51A and 51E (12 per cent), 51A and 51B (4 per cent), 51B and 51E (3 per cent), 51A and non-reactive

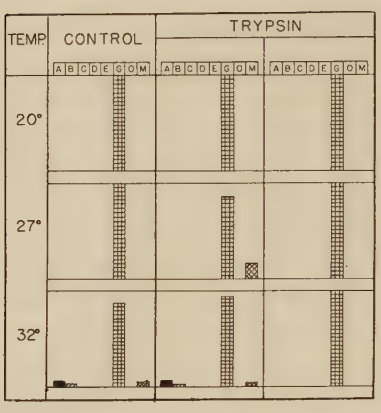

Fig. 6. Transformation of type 51 G after exposure to trypsin for 12 and 24 hours, followed by growth in single isolations for approximately 8 fissions at 20°, 27° and 32°. The length of each bar represents the percentage of the surviving clones that have transformed to the type indicated by the shading.

animals (2 per cent), 51E and non-reactive animals (1 per cent). Those resulting from the 24 hour treatment comprised 51A and 51E (8 per cent), 51A and non-reactive animals (4 per cent), 51A and 51G (1 per cent), 51E and 51G (2 per cent), and 51A, 51B and 51E (2 per cent). Type 51E transforms to a great extent to type 51A when grown at 32°. At this temperature a large number of mixed clones appeared in the control, consisting of the following mixtures: 51A and 51E (15 per cent), 51A and 51B (6 per cent), 51A, 51B and 51E (7 per cent), 51B and 51E (2 per cent). Exposure of this type to trypsin for 12 and 24 hours resulted in the transformation of a large number of clones to type 51A. A smaller number of type 51G appeared also. The exposure to trypsin for 24 hours gave rise to a small number of 51B clones. Only a very small number of mixed clones appeared. These consisted of mixtures of 51A and 51B animals. Exposure to chymotrypsin resulted in an almost complete transformation to type 51A.

Type 51G is stable at 20° and at 27°. Only at 32° some transformation to type 51A (5 per cent) and to type 51B (1 per cent) takes place. The mixed types resulting from the treatment with trypsin for 12 hours, followed by growth at 27° consisted of animals of type 51G and non-reactive animals (6 per cent), and animals of type 51G and type 51E (3 per cent). The mixed types resulting from the treatment with trypsin for 12 hours, followed by growth at 37° consisted of animals of type 51G and non-reactive animals (1 per cent).

The results obtained with the mass cultures are in general agreement with

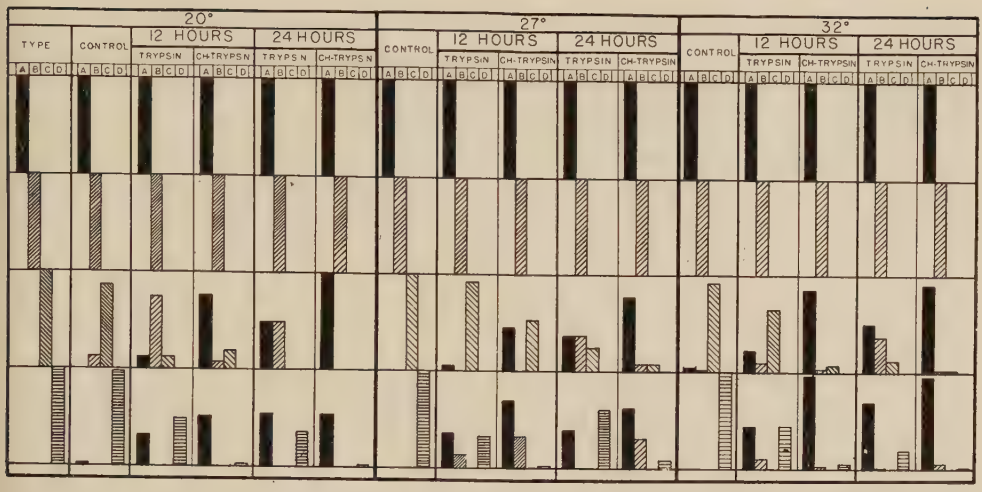

Fig. 7. Transformation of antigenic types 51 A, 51 B, 51 C and 51 D after exposure to trypsin and chymotrypsin for 12 and 24 hours, followed by growth in mass cultures at 20°, 27° and 32°. The length of each bar represents the percentage of the mass cultures that have transformed to the type indicated by the shading.

those obtained with the single isolations (Figure 7), although there is a lesser diversity of types to which the enzyme treated animals transform. No change in antigenic type could be detected in types 51A and 51B. Treatment of animals of type 51D again gave rise to a number of animals that did not react to any of the available antisera.

DISCUSSION

The most striking aspects of the effects of treatment with the two proteolytic enzymes are: [1] No visible effects are detectable in type 51A by treatment with either trypsin or chymotrypsin; [2] type 51B is relatively stable when the enzyme treated isolations are grown at 20° and at 27° (in mass cultures type 51B is stable under all conditions); [3] exposure to trypsin and chymotrypsin results in the appearance of a number of clones of type 51A in the single isolations. This number is always larger than in the non-treated isolations; [4] treatment with chymotrypsin gives always rise to relatively more clones of type 51A than does treatment with trypsin; [5] type 51D transforms under the influence of the enzymes to type 51J.

These effects could be the result of anyone or more of several actions of these enzymes. First, the enzymes could have caused a general injurious effect upon *P. aurelia,* resulting in a change in internal conditions which

could result in a suppression or retardation of the multiplication of the primary antigen. Then one of the other antigens could replace the suppressed one. It is obvious that there is a general injurious effect as indicated by the high mortality during and after exposure to the enzymes. It is reasonable to assume that the mortality rate is correlated with the degree of injury or disturbance in the survivors. Hence, if transformation is a consequence of general injury, then the rate of mortality should be correlated with the frequency of transformation. Type 51A and type 51G have a mortality rate after a 24 hours exposure to trypsin equal to that of other types. Nevertheless, no transformation is evident. Type 51B has a uniformly high mortality in both enzymes, regardless of the duration of exposure. It then ought to show a high rate of transformation. It is however relatively stable. It is also difficult to see how a generalized effect on the viability of *P. aurelia* could cause a differentiation between the effects of trypsin and chymotrypsin. In each instance where transformation takes place a larger percentage of the animals transforms to type 51 A after chymotrypsin treatment than after trypsin treatment. It seems therefore that a general injurious effect of the enzyme treatment can be excluded as the main cause of the observed transformations.

Second, only those individuals of the enzyme-sensitive types which during the exposure to the proteolytic enzymes were able to transform to enzyme-insensitive types might have survived the effects and after effects of the exposure. If this were the case, the surviving clones should belong predominantly to those antigenic types that show the lowest lethal effect during and after exposure to trypsin or chymotrypsin. It can be seen from table I that animals of type 51B are very susceptible to the action of trypsin and chymotrypsin, both after a 12 hours and a 24 hours exposure. In all four instances the total lethal effect of the enzyme action is close to 100 per cent, and in every case higher than the total lethal effect of these enzymes on type 51A. Consequently, type 51B should have been preferentially eliminated over type 51A whenever type 51B arose. This is not the case, as can be seen from Figure 3. The formation of type 51B is pronounced in type 51C, especially after a 12 hours exposure to trypsin. At 20° and at 27° more type 51B arise than type 51A. The postulate of differential survival would therefore not explain the transformations observed after enzyme treatment.

Third, the proteolytic enzymes could have brought about a change in the equlibrium conditions of the reactions leading to the synthesis of the various antigens. Any upset of this equilibrium sets in motion a chain of reactions leading to a new equilibrium state. The pattern of transformation that

results from the exposure to the proteolytic enzymes is similar to those obtained under other experimental conditions, such as exposure to antiserum or temperature (3). It seems therefore likely that there is some common denominator underlying the induction of transformations by these various agents. The nature of this common denominator is as yet unknown. However, the fact that treatment with proteolytic enzymes can induce transformation indicates that a protein must be part of this denominator.

SUMMARY

Exposure of *P. aurelia* to trypsin and chymotrypsin results in the death of a large percentage of the population. Not all antigenic types are equally affected; antigen types 51B and 51E are most affected by exposure to trypsin, while antigen type 51B is most affected by the action of chymotrypsin. Type 51A does not transform when exposed to either trypsin or chymotrypsin, or after the animals have been removed from the enzyme preparations. Type 51B shows little transformations. Types 51C, 51D and 51E transform readily during and after exposure to these two enzymes. In every instance exposure to the enzymes, followed by growth at 20°, 27° or 32° results in the transformation to more type 51A than growth at these temperatures alone. Exposure to chymotrypsin leads invariably to more transformation to type 51A than does exposure to trypsin. Exposure of type 51D to either trypsin or chymotrypsin gives rise to type 51J. The obtained results are analogous with those obtained by transformations, resulting from exposure to antiserum or exposure to different temperatures.

REFERENCES

1. Kimball, R. E., *Genetics*, **32**, 486 (1947).
2. Lancefield, R. C., *J. Exptl. Med.*, **78**, 465 (1943).
3. Sonneborn, T. M., *Heredity*, **4**, 11 (1950).
4. van Wagtendonk, W. J., *Federation Proc.*, **8**, 262 (1949).

THE ELECTRON MICROSCOPY OF ONION ROOT TIP CELLS

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INTRODUCTION

The development of techniques for cutting sections thin enough for electron microscopy now permits serious study of the macromolecular structure of many healthy and diseased cells. An important aspect of such a study is necessarily concerned with cell division. Using both plant and animal material, this has been for some time under study in this laboratory. The present paper describes, as a part of this broader investigation, observations made on root tip cells (3) of the onion, *Allium cepa.*

Before full profit can be derived from our ability to examine sectioned cells with the high resolution of the electron microscope a prolonged study must be made of the degree of damage to their macromolecular structure caused by the usual cytological fixatives. Where such damage is perceptible, better methods of fixation must evidently be sought. It is of course to be expected that all chemicals capable of fixing tissues will bring about chemical changes in them; the significant question is whether or not these changes are extensive enough to alter the visible structures. Obviously the high resolution of the electron microscope will reveal changes that could not have been seen under the optical microscope. Since there is no other way of observing equally minute detail, a recognition of such minimal artefacts must result from electron microscopic observations only. In general this must involve comparisons between the appearance of an object prepared in several different ways. Ultimately widely acceptable criteria for selecting the best methods of specimen preparation will result but experience is not yet adequate to establish them. In the meantime tentative standards must be employed. For this work we have chosen to consider as better and as probably more nearly reproducing the original texture, a fixative that leaves behind a continuous, unbroken pattern of fine detail. Such a basis for judgment has seemed to us preferable to one that seeks to make the familiar appearance of material under the optical microscope the standard for judging its electron microscopic appearance. There are

several reasons for this. For one thing damage and breaks in structure that are missed in the thick optical sections become glaringly evident even under the optical microscope in the 30 to 100 times thinner sections needed for electron microscopy. For another thing there is a natural tendency to prefer a method of preparation which gives a maximum of clarity and sharpness to the finest detail seen in the optical microscope; but study with the electron microscope shows that this clarity is sometimes attained through the coagulation and aggregation of sub-optical microscopic detail. It has been objected that a cellular structure which is a fine-grained continuum (like that of Figures 2 and 3 for example) cannot appropriately be considered as nearer that of native protoplasm than a coarse one (like that of Figures 1 and 18) inasmuch as a fixative could conceivably act as a dispersing agent rather than as a coagulant. The reason for discounting this objection in the present work is the fact that a fixative which yields a protoplasmic texture like that of Figure 3 will produce a coarser one approaching that of Figure 1 if permitted to act for a longer time.

METHODS

The preparations on which the present paper is based were made from onion (*Allium cepa*) root tips that were immersed in a fixative for a given length of time, dehydrated, embedded in methacrylate and sectioned (1) by the thermal expansion method. Sections of suitable thinness were placed on formvar-covered grids, the methacrylate was dissolved away with amyl acetate and the residual tissue was lightly shadowed with palladium or nickel before examination with an RCA type EMU electron microscope.

Ethyl alcohol and pyridine were used for dehydration. The usual passage of a tissue through alcohols of diminishing water content has frequently brought about an undesirably great shrinkage. In this study better results have been obtained when pyridine has replaced alcohol. The shrinkage has been less and though the pH has been around 7.5 during early stages when the water-content was high, the continuity of the protoplasmic texture has been well preserved.

For fixation (2) several of the usual reagents have been used singly and in combination with one another. Flemming's solution was employed at the outset. It quickly became clear that this was causing changes in fine structure that were conspicuous at electron microscopic magnifications. Several variants of this solution which differed in the relative amounts of their component acetic, osmic and chromic acids, accordingly were investigated.

Then the three acids were studied separately. Acetic acid proved to be the most active. Brief contact with tissues, both by itself and in combination with formaldehyde, preserved the optically visible cellular structures but no continuity of texture remained when seen at electron microscopic magnifications. Damage was less obvious when fixation was made with osmic acid. Small pieces of tissue fixed in it for periods up to about half an hour have yielded preparations with fine-grained continuous protoplasmic textures like those shown in this paper. Longer fixation and expecially the usual 24 hour immersion has resulted in a disruption of this texture. Osmic acid has not been satisfactory as a fixative for onion root tips both because its normally slow penetration into tissue is exaggerated by the impermeability of the root epidermis and also because it has rendered the tissues undesirably "opaque" under the electron microscope.

Chromic acid has produced the same disintegration and coarse aggregation of tissue elements visible under the electron microscope as has acetic acid. Fixation with weak neutral bichromate, on the other hand, has given continuous fine-textured preparations and, as indicated below, has been found useful.

Results obtained with formalin as fixative have been strongly affected by both pH and the length of fixation. Acid formalin has produced the same coarsely shredded preparations as have other acid mixtures or dilute acetic acid alone. Fixation with a weak (e.g. 4 per cent) neutral formalin solution leads to a continuous fine-textured protoplasm if limited to about two hours. Longer exposure gives rise to a progressive coarsening and break-up of this texture.

As a consequence of such experiments as these with various fixatives, most preparations of onion root tip have been fixed for ca 2 hours with a 4 per cent neutral aqueous solution of formalin sometimes containing a small amount (ca 0.3 per cent) of $K_2Cr_2O_7$. Except for the two that have been included to illustrate what is seen after acid or prolonged fixation, the electron micrographs that follow have been made of sections fixed in this fashion.

These photographs, typical of about 300 made of this root tip material, have been arranged to illustrate successive phases of the mitotic cycle. As such they provide an introduction to what the electron microscope can show about the finer detail of chromosomes and other structures present during division. It will of course require extended investigations of animal as well as of other plant tissues to supply the information needed for an adequate description and understanding of the macromolecular structures and changes that characterize dividing cells.

RESULTS

Resting cells are shown in the first group of Figures 1 to 4. Of these Figure 1 was made from a preparation fixed in Flemming's solution. In the very thin section needed for electron microscopy all the cellular cytoplasm that remains is a coarse, torn network attached in places to the cell wall and to the nuclear membrane; with the far thicker sections used for optical microscopy this fragmentation would not be so apparent. The nuclei in this figure contain numerous opaque, irregularly shaped clumps of chromatin bound together in the coarse mesh. In many nuclei fixed in this way the accumulation of material around their outer borders creates the impression of thick membranes.

The totally different texture of the protoplasm after neutral fixation is illustrated in Figures 2 and 3. Even in these ultra-thin sections the cytoplasm is not broken up but appears as a fine-grained, uniform network. Within the nucleus the karyolymph has a texture that is equally uniform though always coarser than that of the cytoplasm. It is important that, though the boundary between nucleus and cytoplasm is very sharp, no thick nuclear membrane is visible. Comparison with Figure 1 suggests that the thick membranes sometimes described have been accumulations of debris at the nuclear interface. The chromatin of these resting nuclei occurs in lumps and strands of different sizes. It is extremely opaque under the electron microscope but there are places in Figure 3 where one can see through it. The basic network of the protoplasm continues uninterruptedly across such more transparent regions and it can be seen that the great opacity is due to a material of unresolved fine structure impregnating this net. Nucleoli are equally opaque and appear to have the same structure. Some are quite round in section; others, like those in Figure 3, have deep pockets that are electronically more transparent. Many of our electron micrographs of cells in early prophase suggest that the nucleolus at this time disappears by a process of fragmentation.

The cytoplasm of cells in the actively growing part of the root tip usually has shown few vacuoles. Cells immediately under the epidermis are more vacuolated and their cytoplasm also has contained numerous objects that have the dimensions of "mitochondria." These appear in many of our photographs. They are numerous immediately above the chromatin of Figure 5 and at both the right and left sides of Figure 9. As seen at higher magnification in Figure 4 they are networks still finer in texture than cyt-

oplasm. Their boundaries are very sharp but they do not seem to have enveloping membranes.

Examination of sections through several root tips will reveal cells in all stages of division. These are readily recorded in electron micrographs though photography must be at about the lowest magnifications usually attained with the instrument if complete cells are to be shown. Such electron micrographs of cells in several stages of division are shown in Figures 5 through 12. The cells in rapidly growing root tips often pass from one division to the next without the thorough dispersal of their chromatin illustrated by the resting cells of Figure 2. In such cases it is impossible (Figure 12) to say where late telophase ends and early prophase begins. In cells in this state the aggregated chromatin seems to be coarsely rope-like. It is difficult to gain knowledge of the true shape of telophase and prophase chromosomes from study of the very thin sections required for electron microsocpy but as pictures taken at higher magnifications (Figures 13–15) make clear, their fine structure appears distinctly fibrillar. During early prophase there is a well-defined boundary between nucleus and cytoplasm whether or not the cell has first attained a resting stage. Sometimes one can observe (Figures 5 and 14) cells which are assuming the same overall texture through the disappearance of this boundary.

The photographs thus far made have not illustrated all the steps by which chromosomes develop from the resting chromatin nor have they revealed more than a few stages in the doubling and subsequent separation of the chromosomal pairs. Pairing is well defined in Figure 7 and in a more magnified view of the same chromosomes (Figure 15). The very tenuous nuclear network at this stage (Figure 7) makes it especially difficult to be sure how much of the filamentous structure of the chromosomes of these cells (Figure 15) is artefact introduced by damage to so dilute a gel. Such a filamentous structure is no longer visible when the cell has attained metaphase (Figures 16 and 17). These photographs at high magnification demonstrate the chromatin of these chromosomes as an electron microscopically structureless material impregnating a spongy net-work continuous with that of the rest of the cell. None of the many electron micrographs we have made of metaphase or anaphase chromosomes of the onion has given indication of chromonema, matrix, pellicle and chromomeres; neither is there evidence in such chromosomes of morphological entities that could be recognized as genes.

Many anaphase figures appear in the sections. If the fixative used has been Flemming's or another of corresponding acidity a distinct spindle is

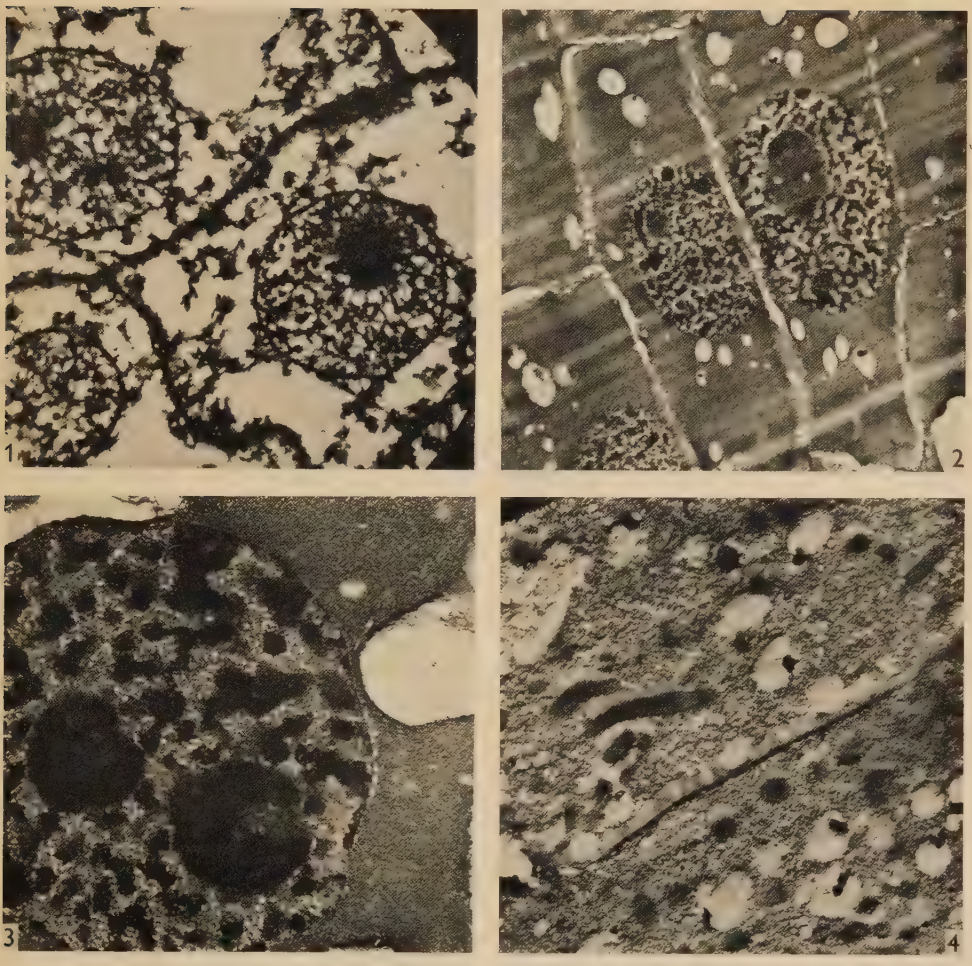


Fig. 1. An electron micrograph through a section of onion root tip fixed in Flemming's solution. It shows portions of three resting cells with their nuclei. Magnification = 4500 ×.

Fig. 2. A section fixed in neutral formalin showing practically all of two resting cells and parts of several others. In contrast to Fig. 1 the protoplasm both within and without the nuclei has a uniform, continuous texture. 4300 ×.

Fig. 3. Part of a single resting cell at higher magnification. Fine structure is visible within the two nucleoli and the chromatin masses of the nucleus. Note the more open texture of nuclear protoplasm compared to that of the cytoplasm. 13 000 ×.

Fig. 4. Part of the cytoplasm of a cell showing many "mitochondria" of various shapes and sizes. The especially fine texture of their protoplasm is clear. 9500 ×.

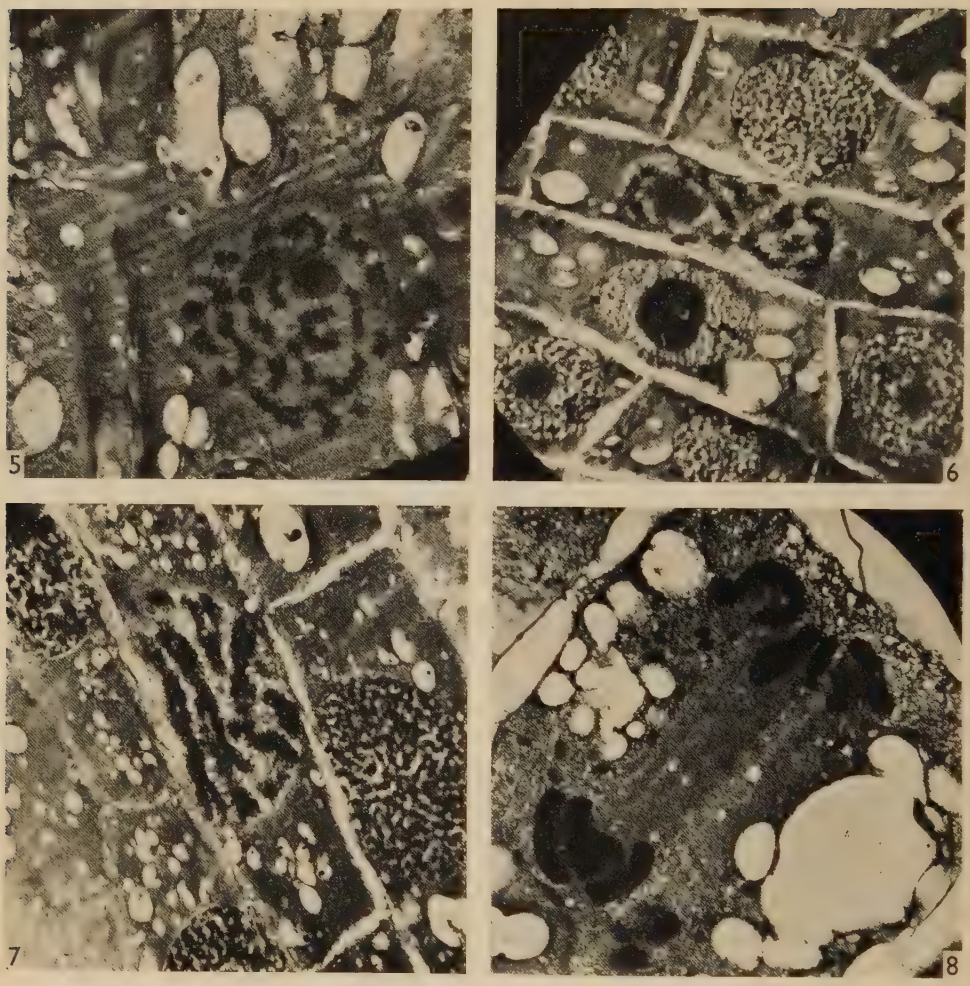
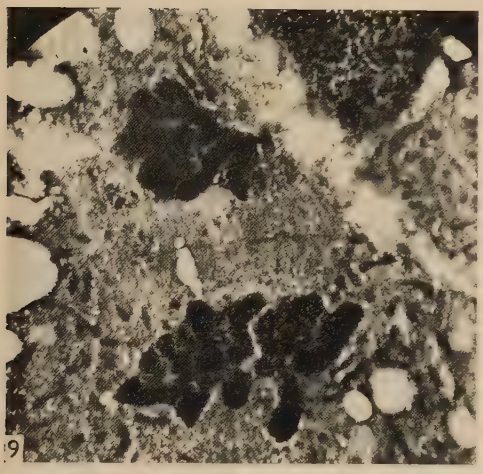


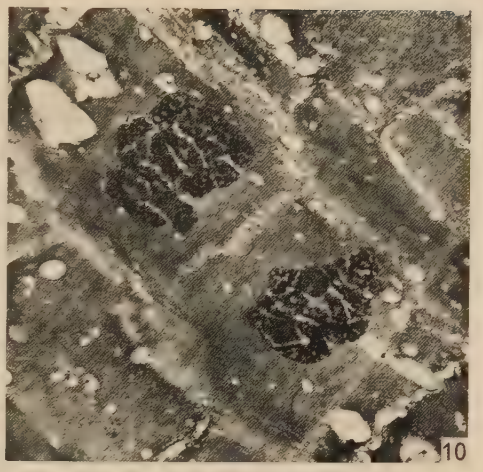
Fig. 5. Part of a cell in prophase. The chromatin has associated to begin formation of chromosomes and the boundary between nucleus and cytoplasm is disappearing. Numerous "mitochondria" are to be seen immediately above the chromatin mass. 4300 ×.

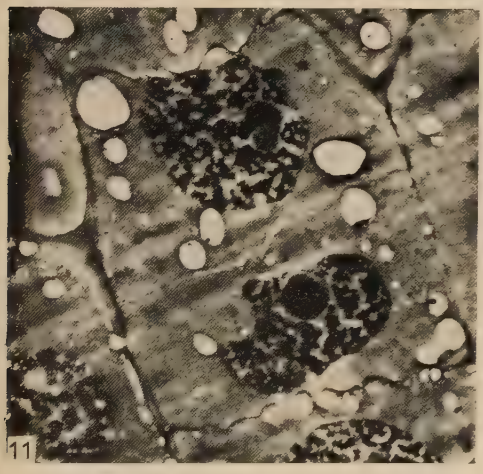
Fig. 6. Several cells of onion root tip. Chromosomes are well developed in the prophase cell at the center. 4300 ×.

Fig. 7. Groups of paired chromosomes are evident in the central cell of this figure. They are seen at higher magnification in figure 15. 4300 ×.

Fig. 8. A cell in anaphase. Note the denser protoplasm seemingly stretched between the chromosomes and the "mitochondria" on either side of it. 5000 ×.







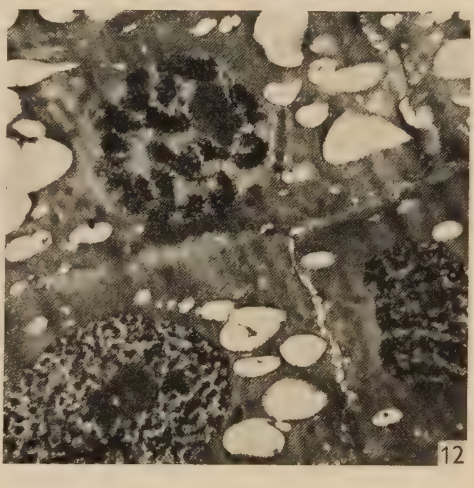
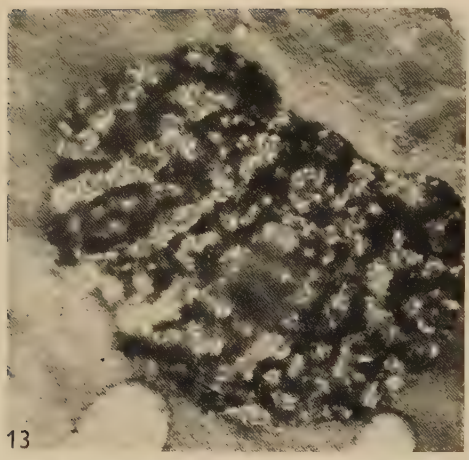


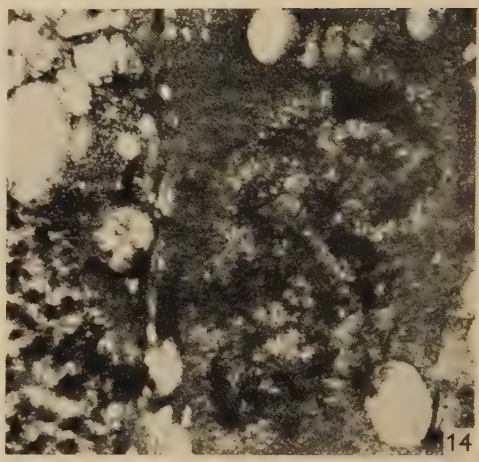
Fig. 9. A cell in telophase. The middle lamella is here visible as a thin dark line with a cluster of "mitochondria" at either end. 4300 ×.

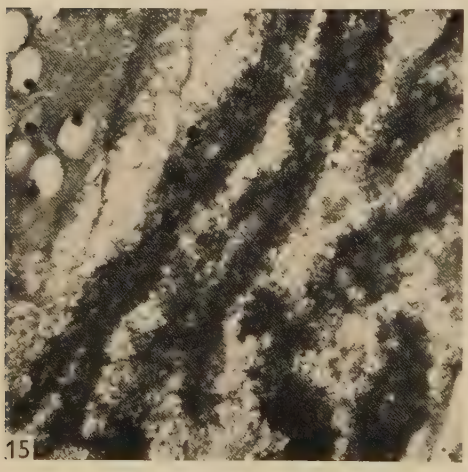
Fig. 10. Another cell in telophase. Here the position of the middle lamella is taken by a series of fine vacuoles where the daughter cells are beginning to part. 4300 ×.

Fig. 11. A cell in the slightly more advanced telophase where the daughter nuclei are well delineated and have developed nucleoli. 4300 ×.

Fig. 12. Parts of three cells. The nucleus at the bottom left is resting, that showing in part at the extreme right is in telophase. The nucleus above is in prophase and has its chromatin aggregated to form chromosomes. 4300 ×.







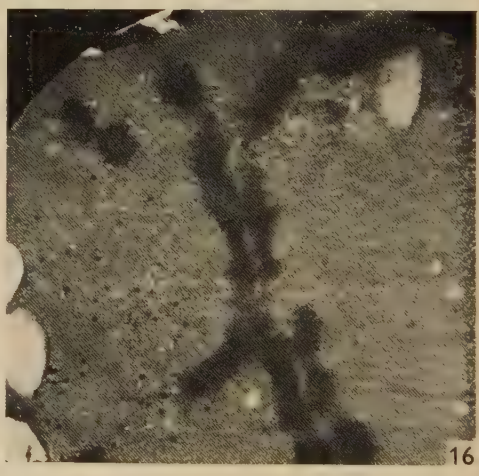
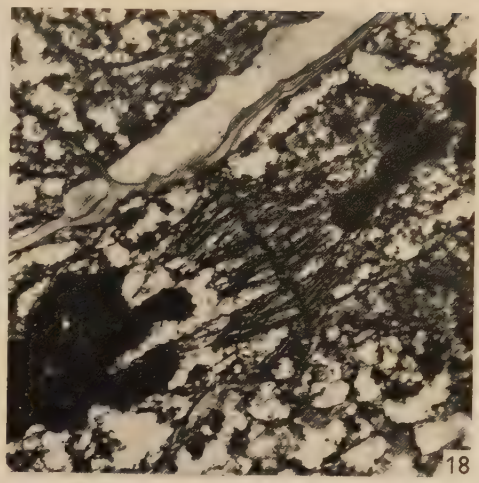


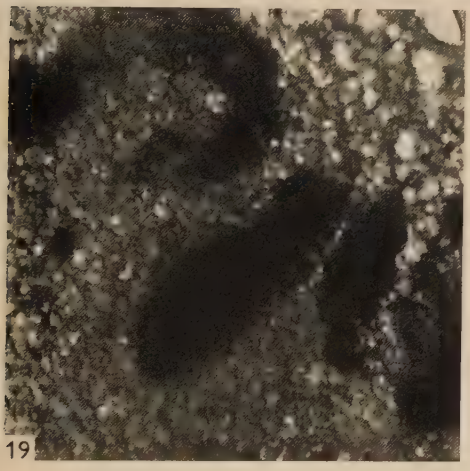
Fig. 13. Nucleus of a telophase cell at high magnification. The filamentous character of the chromatin is apparent. 13 000 ×.

Fig. 14. A prophase nucleus at higher magnification. The chromatin here is even more delicately filamentous than in the preceding figure. 13 000 ×.

Fig. 15. Some of the paired chromosomes of the cell of Figure 7 photographed at higher magnification. The strands composing them are readily discernible. 13 000 ×.

Fig. 16. A section through well-paired chromosomes of a cell in metaphase. The filamentous structure of the chromatin illustrated in the preceding photographs is absent here. 10 000 ×.





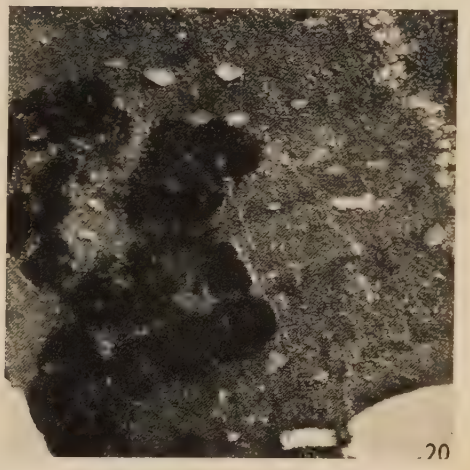


Fig. 17. Metaphase chromosomes at relatively higher magnification. Their greater opacities around their borders and the continuity of the underlying gel structure across these borders are noteworthy. 13 000 ×.

Fig. 18. A cell in anaphase after fixation with Flemming's solution. The spindle and the opaque disk traversing it are wellmarked. 8000 ×.

Fig. 19. One group of the anaphase chromosomes of Figure 8 photographed at higher magnification. Note how the substance of the "interrogation point" chromosome impregnates the underlying protoplasmic net. 16 500 ×.

Fig. 20. Half of a telophase cell at higher magnification showing the fibrillar structure of the retracting chromosomes. Vacuoles traversing the cell at the position of the middle lamella appear at the extreme right. 14 000 ×.

often visible stretching between the polar groups of chromosomes. This is usually bisected by a more or less complete disk through the equator (Figure 18). Figure 9 shows the closest approach to such a figure we have obtained in neutral-fixed preparations. A delicate lamella is visible and the spindle is evident as a preferred orientation or polarization of the strands of the protoplasmic network. It is much less pronounced than the spindle of Figure 18 and is more the sort of thing one would expect to find from a gel under tension. Possibly the coarse strands making up the spindle of Figure 18 are, like other features of acid-fixed preparations, thick aggregates resulting from partial rupture of the native gel. Though most cells in anaphase fixed in neutral-fixatives have not shown a definite equatorial disk, there is often a preferred orientation and a slightly increased opacity of their interchromosomal protoplasm (Figure 8). This too suggests a gel under tension. At telophase when the chromosomes are rounding up to form new nuclei, a series of tiny vacuoles is often seen across the cell in the position of the middle lamella (Figure 10). It is likely that the pectin which composes it at this period is extracted during the specimen preparation.

Anaphase chromosomes, when seen at higher magnification (Figure 19), have consisted of the same kind of homogeneous material impregnating the protoplasmic net that is seen with metaphase chromosomes (Figures 16 and 17). The loss of this homogeneity at telophase (Figure 10) is more evident at the higher magnification of Figure 20.

DISCUSSION

The experiments described in the preceding paragraphs and illustrated by the electron micrographs that accompany them cannot be considered as more than a first step towards gaining an understanding of the macromolecular texture of the protoplasm of plant cells and of the chromosomes that form in them during cell division. These experiments do show that electron microscopic techniques of specimen preparation have advanced to the point where it is profitable to study these fine structures and they illustrate the wealth of new detail that can be seen in this fashion. The great differences at electron microscopic resolutions in the appearance of the onion root tip cells after acid and neutral fixation are striking. They emphasize the need for extensive experimentation with many chemicals to provide a background for efforts to pass from what is seen in the electron microscope to the protoplasmic and chromosomal texture of living cells. Obviously work

with many other types of cells, and with various procedures useful in specimen preparation, should precede any serious attempt to offer such interpretations.

SUMMARY

A series of electron micrographs has been made of thinly sectioned onion root tip fixed with several types of fixative and embedded in methacrylate. With acid fixation the cellular protoplasm appears shredded at these high magnifications, but after neutral fixation the cellular contents form continuous networks that indicate a better state of preservation at macromolecular dimensions. The electron micrographs show the fine structure of the protoplasm and chromosomes of cells in a number of stages of division. Prophase and telophase chromosomes appear to have a fibrillar structure which is no longer visible at metaphase and anaphase.

REFERENCES

1. Newman, S. B., Borysko, E., and Swerdlow, M., *Science*, **110**, 66 (1949).
2. Rozsa, G., and Wyckoff, R. W. G., *Biochim. et Biophys. Acta*, **6**, 334 (1950).
3. Since this paper was written the following paper containing electron micrographs of onion root tip cells has come to attention:
   Bretschneider, L. H., *Proc. Koninkl. Ned. Akad.*, **53**, 1476 (1950).

STUDIES ON ENZYMES IN THE DEVELOPING SEA URCHIN EGG

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Runnström's conception of the double gradient system has played a prominent role in the discussions on the developmental physiology of the sea urchin egg. This conception is based on the remarkable observations of Herbst (43–46) on the influence of lithium ions and sulphate free sea water on the development of the sea urchin egg, on Hörstadius' operative results (54), and on Runnström's studies (103) on the sensitivity of different parts of the eggs to CO. According to this conception, which was gradually developed in a series of papers (102–106) two antagonistic gradients exist in the direction of the egg axis. Runnström (106) postulated that these gradients correspond to animal and vegetal substances produced through an animal and vegetal type of metabolism. The Li ion, which favours the development of the entoderm at the expense of that of the ectoderm, should check the animal type of metabolism.

Hörstadius (51–55), and Lindahl (73–78) have presented many facts in support of this hypothesis. Lindahl (78) studied the metabolic background of the determination. He focused attention on the respiratory changes which take place in the developing egg, and found these changes to be sensitive to Li treatment. Since differentiation must depend upon different kinds of synthetic processes in different parts of the egg, one of the present authors has been making a study of the protein metabolism of the developing egg. The results from various kinds of investigations have hitherto stressed that the cleavage stages, during which the primary determination of the germ layers proceeds (53, 75) are rather uneventful as regards changes in amino acid metabolism, serological and enzymatic properties (38, 39, 40, 86). As a rule such changes do not occur until around the onset of visible differentiation, which may be defined as the appearance of the first mesenchyme cells at the vegetal pole of the blastula. These results have led Gustafson (37) to the assumption that the synthetic processes on which determination is based are not very elaborate. He followed the idea of Runnström (108) that small populations of protein molecules of new specificity might be built up during

the period of determination. At the onset of differentiation, when conditions for rapid protein synthesis appear, it is assumed that a rapid growth of these populations takes place. He also expressed the hypothesis in more cytological terms, i.e. that the animal and vegetal compounds should control the growth of some populations of self-reproducing particles, presumably of nucleoprotein nature. The studies on the synthetic processes have been continued to get a further insight into these problems. The enzymatic studies have constituted an essential part of this series of investigations since the enzymes are easily characterized proteins and are themselves tools for further syntheses. One may suppose that the differences in enzymatic activity between homogenates of different developmental stages may on the whole reflect the actual changes in enzyme content in the cell.

MATERIALS AND METHODS

In the present work the following species of sea urchins have been used: *Paracentrotus lividus* and *Sphærechinus granularis* from the Gulf of Naples, and *Psammechinus miliaris*, *Echinocardium cordatum*, *Strongylocentrotus dræbachiensis*, and *Echinus esculentus* from the West Coast of Sweden. The use of different kinds of material with a very different temperature optimum of development has to some degree made it difficult to draw conclusions about the exact relation between the changes in activity of the different enzymes. On the other hand the use of numerous different species has given the impression that the main changes in enzymatic activity are generally not very much dependent upon the species used, as long as the changes in activity are referred to the stage of morphological development.

After artificial fertilization, the eggs were cultivated according to the method of Lindahl (73). For Li treatment the homogenous egg suspension was divided into two equal parts immediately after fertilization. These parts were then cultivated separately. One of these was used as the control. At an early cleavage stage the eggs of the other tray were allowed to sediment and 10–12 per cent of the sea water was removed without any loss of eggs. The same volume of isotonic LiCl solution was then added and the eggs were cultivated further for three to five hours. The exact time of the Li treatment is indicated on the separate curves below. After Li treatment the eggs were washed and transferred into normal sea water. The final volume was the same as that before Li treatment.

At different times of development samples were taken as described in an earlier paper (38). The eggs were, however, generally washed with Ca-free sea water or isotonic KCl solution before the last centrifugation in order to facilitate the breaking of the cell walls. In certain cases the washed eggs were treated for 5 minutes at 0° C with an equal or double volume of glass-distilled water or 0.05 molar phosphate buffer in order to make the cells swell and thus make homogenization easier. The suspension was then vibrated on an electric vibrator at 50 cycles per second for one to three minutes. After this treatment the cells were completely or almost completely

disintegrated. The samples were then rapidly frozen by using a freezing mixture of NaCl and ice or dry ice and alcohol or more slowly in a refrigerator box at 20° C below zero. The samples were generally stored for a couple of days in the refrigerator box after which time all samples were analyzed simultaneously. In the case of e.g. apyrase, the analyses were run directly after homogenization since storage was found to reduce the activity somewhat. According to our experience the same drawback seems to prevail also in the case of malic dehydrogenase. Storage causes a dissociation of the apo- and coenzyme from the mitochondria which has a detrimental effect on the activity (59).

The eggs were generally vibrated again while thawing. This vibration was often necessary for breaking the fertilization membrane, inside which the disintegrated cells were situated. The vibration was sometimes supplemented with a grinding of the thawing sample with a glass rod, and in some series of experiments the samples were homogenized merely by grinding the thawing samples with a glass rod. In this case thawing was prolonged by occasional cooling using a freezing mixture. In each series of estimations all samples, irrespective of the developmental stage, were homogenized in the same manner. The effect of homogenization was always carefully checked in the microscope. Incomplete homogenization, which occurred rather frequently in preliminary experiments, may cause an apparent change in activity during e.g. cleavage stages. It is, however, important to stress that the nuclei were not completely disintegrated but had a wrinkled appearance. It is to be assumed that enzymes present in the nuclei may diffuse out (24) and substrate diffuse into the nuclei. This may at least hold true for alkaline phosphatase which has been considered to be bound to a great extent to nuclei (69) and shows a strong rise at gastrulation (38, 86) as follows from measurements on homogenates with the present technique of homogenization. Certain enzymes, however, are reported to show a low apparent activity if the nuclei are not completely disintegrated (24).

The homogenized samples were generally kept in an ice bath for a few minutes before analysis and were diluted with buffer, isotonic KCl solution, distilled water, or sometimes with isotonic NaCl or even sea water. The dilutions were mixed with buffer as soon as possible, since acidification occurs at homogenization. The final pH was always carefully checked with a Beckman pH-meter with glass electrode. The temperature of incubation varied from case to case and was never above 30° C and generally at 23–25° C.

Since the aim of this work is not to study the kinetics of the different enzymes but to get a general view of the changes in enzymatic activity which occur during development, the studies of the optimal conditions for the enzymatic activity have been rather limited. The modifications of current methods for the determination of enzyme activity have generally not changed the final composition of the reaction mixture described. We have as a rule checked the range where the activity was directly proportional to the time of incubation and to the amount of enzyme in serial dilutions. The pH optimum has generally been determined and the enzymatic activity has been assayed at the optimal pH.

As the main purpose of this work consists in a comparison of the activity of the same enzyme in different developmental stages, only relative values of activity have been recorded. Our technique permits a sampling which gives the same number of eggs at each stage of development. By merely using a factor it would thus be

possible to express the absolute enzymatic activity per egg. For enzymes with a developmental change in activity, the maximum value has been designated as 100. For enzymes with a constant activity throughout development the average activity has been given the value 100.

The good correspondence between the series with slightly modified technique, i.e. mode of homogenization, dilution, buffer used etc., is a criterion that the results are not primarily dependent upon the method of treating the samples.

RESULTS

Apyrase

The term apyrase is used to distinguish the ATP-splitting activity in cells from that known to be due to true ATPase, cf. (30). No attempt was made to differentiate between ATPase and ADPase. The determinations of the apyrase activity used in the present investigation were performed in the following manner. A solution of sodium–ATP was prepared from the di-barium salt of Sigma. Two ml of veronal-acetate buffer, pH 7.4, 1 ml 0.004 M calcium chloride, and 1 ml homogenate in isotonic KCl solution were mixed and kept in a 25° C water bath. After temperature-equilibrium had been reached, 1 ml 0.002 M ATP solution of 25° C was added and the reaction mixture was incubated for 15 minutes, after which the reaction was stopped by adding 4 ml of ice-chilled 10 per cent trichloroacetic acid. The sample was then left for 10 minutes in an ice bath. After centrifuging, orthophosphate was determined in an aliquot by the method of Briggs (17). To controls, which were run simultaneously with the sample, ATP was added immediately before the fixation with trichloroacetic acid. With this method the reaction is approximately linear during the time of incubation and there is a linear relation between concentration in serial dilutions of the homogenates and orthophosphate liberated. The points recorded on the curves are well within the range of a linear relation. Calcium had no clear effect on the homogenates, an observation which has also been reported by Connors and Scheer (21) for sea urchin material and also for some other kinds of material (30, 94). The amount of calcium added was, however, small.

The effect of different media for homogenization and dilution has been tested. Isotonic KCl or NaCl and distilled water gave the same results. The samples were removed from the cultures, washed with isotonic KCl, homogenized and tested immediately, since Meyerhof and Wilson (89) reported that the activity of the apyrase in rat sarcoma is reduced if stored in the frozen state for 1–3 days. A sample of sea urchin eggs stored for 36 hours on dry ice had lost about 25 per cent of its original apyrase activity.

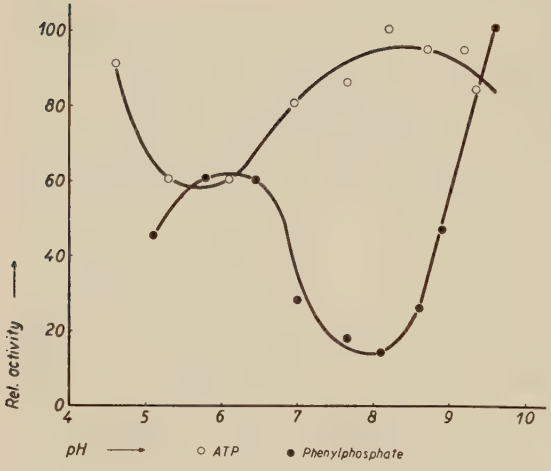

Fig. 1.

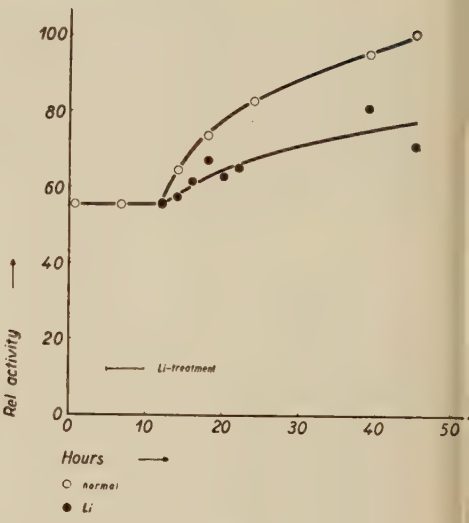

Fig. 2.

Fig. 1. Activity-pH curve of phosphomonoesterases (phenyl-phosphate as substrate) and ATP splitting enzymes. Material: Gastrulae of *Paracentrotus lividus*. Buffer: Veronal-acetate.

Fig. 2. Relative apyrase activity in different normal and Li treated developmental stages of *Paracentrotus lividus*. Primary mesenchyme appeared after 13 hours during normal development and the larvae were well developed plutei at 42 hours. Li treatment (10 parts 0.62 M LiCl to 90 parts sea water during the interval indicated on the curve) gave rise to strongly vegetalized exogastrulae with ectoderm of about the same size as that of the stomach.

The method of freezing and homogenization may appear drastic but according to the authors quoted above the apyrase of rat sarcoma was not significantly reduced even after 2–3 hours in the frozen state. Homogenization by freezing and vibration of the thawing sample 1, 2, and 6 times gave preparations of the same activity. It is important, however, that no small air-bubbles are formed during homogenization, since they may cause clotting of the proteins in the homogenates resulting in a sharp drop in the activity. Therefore, the vibration was generally substituted by grinding the thawing samples with a glass rod.

The pH of the digests was chosen so as to minimize the effect of the acid and alkaline phosphomonoesterases on adenylic acid. According to experiments with homogenates of gastrulae of *Paracentrotus*, in which the pH was carefully checked, the phosphomonoesterases have a minimal activity on phenylphosphate at about pH 8.0, whereas the apyrase has an optimum at the same pH (fig. 1). pH 7.4 was chosen for assay of apyrase, as the acid phosphomonoesterase has a rather constant activity during development,

whereas the alkaline phosphatase changes considerably in activity, at about the time of gastrulation.

The di-barium salt of ATP slowly disintegrates during storage into adenylic acid and inorganic pyrophosphate (6). Since the egg contains an inorganic pyrophosphate splitting enzyme with an optimal activity at about pH 7.4, a certain interference by this enzyme cannot be excluded. Since the activity of this enzyme is constant throughout development a possible interference by this enzyme does not essentially change our conclusions about the apyrase activity.

The results were consistent and the curves recorded are typical examples (fig. 2). The apyrase activity is constant up to the appearance of the primary mesenchyme cells, i.e. to about 13 hours after fertilization for *Paracentrotus lividus* at 22° C. Thereafter the activity increases and was approximately doubled after 48 hours. Li treatment e.g. 10 per cent isotonic LiCl and 90 per cent sea water (0.062 M LiCl) between 2–3 to 6–8 hours after fertilization impedes this rise. Since too strong Li treatment also may cause general disturbances in addition to vegetalization we cannot for the present say if the rise in activity may be checked further after stronger treatment. The authors have, however, the impression that a stronger Li treatment impedes the rise in activity even more completely than the treatment mentioned above.

Pyrophosphatase and metaphosphatase

Pyrophosphatase activity was estimated according to the method of Naganna *et al.* (95) by determination of the orthophosphate liberated during 15 minutes at 20° C from sodium-pyrophosphate in a reaction mixture containing veronal-acetate buffer of pH 7.6. $MnCl_2$ was added as activator and the egg homogenate diluted with glass distilled water. Metaphosphatase activity was determined according to the same method, except that sodium-hexametaphosphate was substituted for inorganic pyrophosphate. Sodium-trimetaphosphate, which is considered to have a cyclic structure (62), was used in the same way, but no activity could be demonstrated with this substrate. The determinations were performed in the range where the amount of liberated orthophosphate is a linear function of enzyme concentration and also of time of incubation. The pH curve from homogenates of *Paracentrotus* eggs is very much the same for both inorganic pyrophosphate and hexametaphosphate. The pH optimum lies between 7.0 and 8.0 in both cases, which is in good accordance with data from other material (fig. 3), (62, 101).

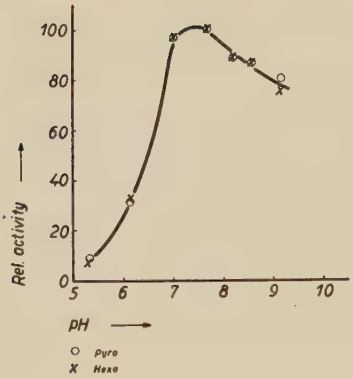

Fig. 3.

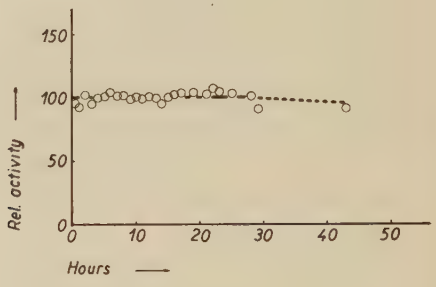

Fig. 4.

Fig. 3. Activity-pH curves of pyrophosphatase and hexametaphosphatase. Material: Plutei of *Paracentrotus lividus*. Buffer: Veronal-acetate.

Fig. 4. Relative pyrophosphatase activity of different normal developmental stages of *Paracentrotus lividus*. Rate of development see text to fig. 2.

The activity, which is of the same order for both substrates, is rather constant throughout the development of *Paracentrotus lividus* (figs. 4, 5). Preliminary experiments on stages up to the age of 20 hours show no effect of Li.

Acid phosphomonesterase

Acid phosphatase activity was essentially determined according to the method of Binkley *et al.* (12) using phenylphosphate as substrate. The digests were buffered by means of acetate buffers of pH 5.4. No Mg was added. Incubation was performed for 1 hour at 23° C. The pH optimum in veronal-acetate buffered homogenates is approximately 6.0 (fig. 1). Determinations at various developmental stages of *Psammechinus miliaris* were made on homogenates of KCl washed eggs diluted to varying degrees with glass-distilled water. They always gave essentially the same result, i.e. the activity is rather constant during development up to the pluteus stage. Li vegetalized larvae showed the same activity as normal larvae (fig. 6). The low activities during the first few hours on the curve recorded may be due to incomplete homogenization. The activity during early development is several times higher than that of alkaline phosphatase which begins to rise at about the mesenchymeblastula stage (38). The same results on normal development of *Arbacia punctulata* have been reported by Mazia *et al.* using glycerophosphate as substrate (86). Preliminary experiments in *Paracentrotus*, however, show a slight but definite developmental rise in activity.

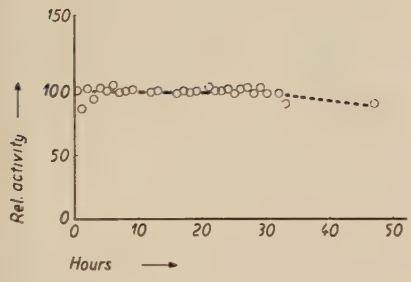

Fig. 5.

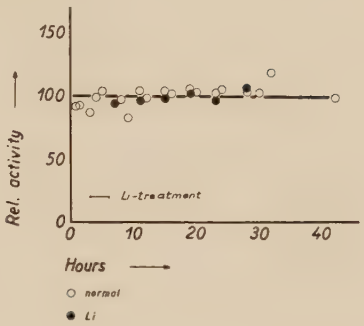

Fig. 6.

Fig. 5. Relative hexametaphosphatase activity of different normal developmental stages of *Paracentrotus lividus*. Rate of development see text to fig. 2.

Fig. 6. Relative activity of acid phosphomonoesterase in different normal and Li treated developmental stages of *Psammechinus miliaris*. Primary mesenchyme appeared after 11 hours and somewhat shortarmed plutei appeared at 40 hours. Li treatment (9 parts 0.53 M LiCl to 91 parts sea water) between 3–6 hours after fertilization gave rise to strongly vegetalized larvae of the same type as described in the text of fig. 2.

Succinodehydrogenase

For the determinations of succinodehydrogenase a simple rapid routine method was sought which could give somewhat more accurate values than the determination of decolorization rates of methylene blue. Kun and Abood (70) describe the use of triphenyltetrazoliumchloride as a hydrogen acceptor. The red, insoluble formozan which was formed could be dissolved in acetone which also precipitates the proteins. The acetone solution is very well adapted for colorimetric measurements. Preliminary experiments with egg homogenates showed that the activity was very low. In the following experiments the composition of the digests was the same as that of Schneider and Potter (109), as described by Flexner and Flexner (32) for gasometric determination of the enzyme activity. Cytochrome C which had no function in our experiments was omitted. By addition of a small volume of triphenyltetrazoliumchloride (0.1 ml of a 1 per cent solution to 2.6 ml final volume) the final concentration of the hydrogen acceptor was about the same as described by Kun and Abood. In order to get strong colors the evacuated tubes were incubated for 1 hour at 25° C. The red pigment which appears during larval development is insoluble in acetone. Carotenoids, however, dissolve easily but this color was compensated for by the blanks. The relation between time of incubation or amount of enzyme and color development has not been studied on sea urchin eggs. The amount of color which may be absorbed by the proteins

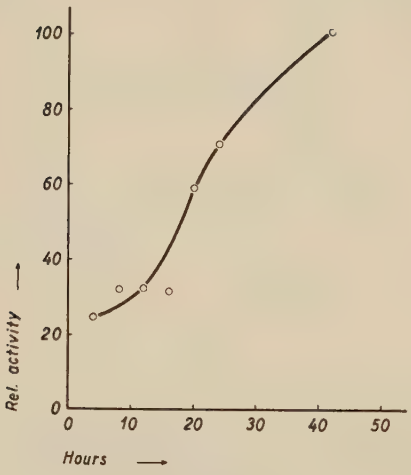

Fig. 7.

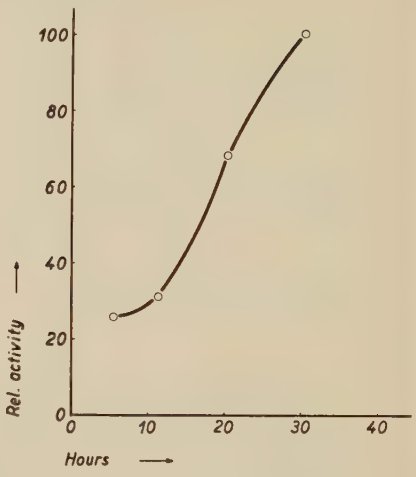

Fig. 8.

Figs. 7, 8. Relative activity of succinodehydrogenase in different normal developmental stages of 7) *Psammechinus miliaris*, 8) *Echinocardium cordatum*. Primary mesenchyme appeared at about 11 hours in *Psammechinus*, cf. fig. 6, and at 9 hours in *Echinocardium*.

even after addition of acetone has not been determined. The authors have the impression, however, that this absorption is not appreciable.

The succinodehydrogenase activity was determined in *Paracentrotus lividus, Sphaerechinus granularis, Echinocardium cordatum,* and *Psammechinus miliaris*. In all four cases the activity is constant up to the mesenchymeblastula stage when there is a strong rise in activity. The curves recorded demonstrate the activity changes in *Psammechinus miliaris* and *Echinocardium cordatum* (figs. 7, 8). No experiments on the color development as a function of amount of enzyme have been made as yet. The rise in activity may thus be greater than appears from the curves. From the curves recorded the constancy of the activity during cleavage stages is not obvious. Experiments on *Sphaerechinus* and *Paracentrotus* are, however, convincing in this case.

Malic dehydrogenase

According to our experience, malic dehydrogenase is very sensitive to various factors such as the method of preparing the homogenates, freezing etc. Huennekens' (59) studies on the factors which influence malic dehydrogenase activity has led us to the following method. We used concentrations

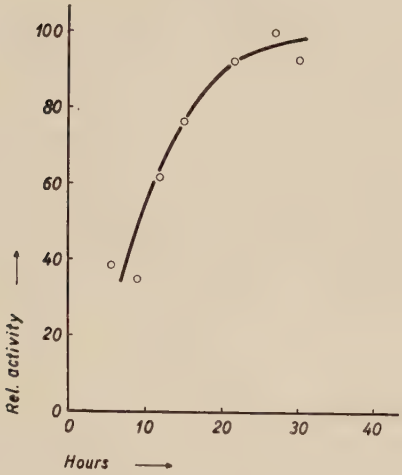

Fig. 9. Relative activity of malic dehydrogenase in different normal developmental stages of *Psammechinus miliaris*. Primary mesenchyme appeared at 9 hours, cf. fig. 6.

of cozymase which permit the determination of the sum of the activity of both the "conjugated" and "dissociated" enzyme. The reaction mixture had the following composition, cf. (59).

0.25 ml 0.20 M Na-l-malate, 0.50 ml 0.13 M phosphate buffer, 0.10 ml 0.06 per cent methylene blue, 0.10 ml 2 M KCN pH 8.0, 0.25 ml 400 γ/ml cozymase, 0.10 ml 0.026 M $MgCl_2$, 0.10 ml 0.018 M adenylic acid, 0.20 ml 0.60 M NaF and 0.50 ml homogenate.

The pH of the mixture was 7.5. Diaphorase was not added. The temperature of incubation was 25° C. The eggs and larvae were washed with isotonic KCl and were then kept for 5 minutes in 1/15 M phosphate buffer of pH 7.0 in an ice bath. The material was then ground for 5 minutes when cooling in an ice-NaCl mixture. The homogenate was immediately used for determining the activity. This kind of homogenization has probably caused a significant dissociation since omission of cozymase or KCN prolonged the time of decolorization by 250 and 200 per cent respectively, cf. (59).

The changes in malic dehydrogenase activity during development were determined in *Psammechinus miliaris*. Here the activity rises at mesenchyme-blastula stage as in the case of succinodehydrogenase (fig. 9). The activity

from fertilization to mesenchymeblastula stage is rather constant, as has been determined in several separate experiments. Preliminary studies on the earlier developmental stages of *Strongylocentrotus drœbachiensis* show the same changes in activity as has been described for *Psammechinus*. As diaphorase was not added to the reaction mixture, the acceleration of the decolorization rate may be due to a synthesis of diaphorase as well. Since both diaphorase and malic dehydrogenase are localized in the mitochondria, cf. e.g. Huennekens (59), it could be expected that the changes in decolorization rate express a synthesis of both components.

Aldolase

Aldolase was determined according to the method of Sibley *et al.* (112). The method was, however, slightly modified. Thus, veronal-acetate was used as buffer instead of trishydroxymethylaminomethane; incubation was made at a lower temperature, 20–30° C, and at pH 7.6 instead of 8.6. After the analyses had been performed the method of Sibley *et al.* was criticized by Dounce *et al.* (25). This criticism, however, mainly deals with conditions which we had already modified. The color developed was directly proportional to the incubation time and to the concentration of enzyme in serial dilutions of the egg homogenates. The blanks contained considerable amounts of chromogenic substances which must be partly derived from the homogenates.

The optimal pH was found to be between 7.0 and 8.0 (fig. 10). This is more acid than that reported by Sibley *et al.* for an undefined aldolase preparation. Dounce has partly explained the high pH optimum found by Sibley *et al.* and reported the value 7.2 both for crystalline aldolase and for muscle homogenate. On the other hand, liver homogenate was reported to give curves with a broad optimal range from 7.0 to 8.6, as we also found for sea urchin homogenates. The abnormally high pH optimum which is obtained with these homogenates may be due to the presence of interfering substances in homogenates which are not sufficiently diluted to give a negligible effect (25).

The interference of phosphotriose isomerase as an explanation of the abnormal pH optimum may be indicated by the following facts: 1) There is a second peak on the pH curves both of crystalline aldolase with trishydroxymethylaminomethane as buffer and also occasionally in liver homogenate (25). 2) The use of crystalline aldolase may not exclude the presence of small amounts of isomerase, cf. (87). 3) The isomerase content is often

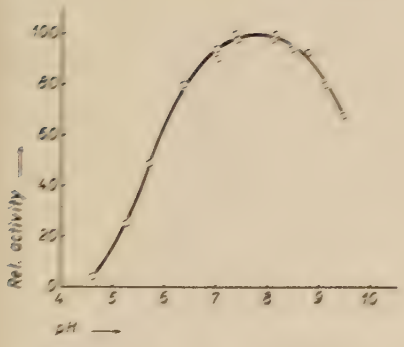

Fig. 10.

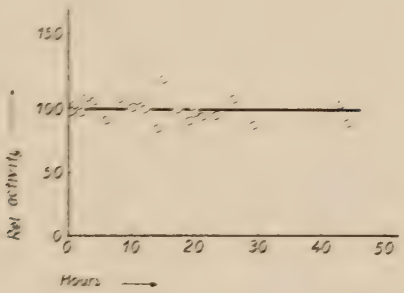

Fig. 11.

Fig. 10. Activity-pH curve of aldolase in ovaria of *Echinus esculentus*. Buffer: Veronal-acetate. Essentially the same curve could be obtained with developmental stages of *Paracentrotus lividus*.

Fig. 11. Relative aldolase activity in different normal developmental stages of *Paracentrotus lividus*. Rate of development see fig. 2.

40-100 times higher than the aldolase activity in tissues (97) and has the same activity at pH 7.0 and 7.8 (97). 4) An incomplete fixation of the triose phosphates by hydrazin has been suggested (25). 5) The color intensity of the dihydroxyacetone derivative is much stronger than that of the glyceraldehyde (112). 6) At equilibrium, the ratio of concentration of dihydroxyacetone phosphate to phosphoglyceraldehyde concentration is about 20 (97, 118, 88).

Determinations on different developmental stages have been made in *Paracentrotus lividus* on homogenates in sea water. The curves show that the activity is constant during development (fig. 11). No studies of Li treated eggs have been performed.

Since interference by phosphotriose isomerase may occur, the results are most correctly described by postulating that the color formation is the same for homogenates from different stages. This may indicate that both enzymes have constant activity throughout development.

Glutaminase

Glutaminase activity was determined in homogenates of KCl-washed eggs, diluted with glass-distilled water. A mixture of 0.5 ml veronal-acetate buffer, 0.1 ml 0.1 M l-glutamine and 0.5 ml homogenate was incubated at 23° C for 2 to 4 hours. The substrate, obtained from red beets according to the de-

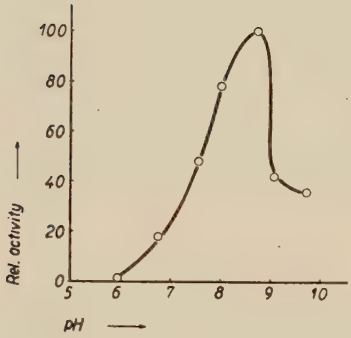

Fig. 12.

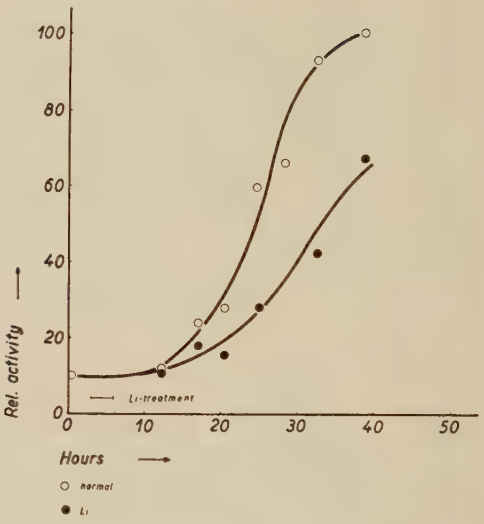

Fig. 13.

Fig. 12. Activity-pH curve of glutaminase in homogenates from plutei of *Psammechinus miliaris*. Buffer: Veronal-acetate.

Fig. 13. Relative glutaminase activity in different normal and Li treated developmental stages of *Psammechinus miliaris*. Li treatment and rate of development see fig. 6.

scriptions in Biochemical Preparations (13), was recrystallized several times. The ammonia liberated was determined by nesslerization after distillation into hydrochloric acid by means of the Conway technique (22) with equal volumes of saturated potassium carbonate and sample. The pH of incubation was 8.7 at which the enzyme had its highest activity (fig. 12). The same pH optimum has been reported for e.g. glutaminase in rat brain extracts which indicates that the enzyme could be classified as a glutaminase I (122). This enzyme is stimulated by phosphate. Calculations on the amount of phosphate present in the digests from eggs of different stages of development indicate that differences in phosphate concentration are not likely to essentially affect our results. A series of determinations of the activity with an excess of phosphate in the digests will, however, be performed. The results on *Psammechinus miliaris* invariably show a constant low activity up to onset of gastrulation after which a strong rise appears. Li treatment during intermediate cleavage stages strongly retards this increment (fig. 13). Experiments on *Paracentrotus* also show a strong rise in activity during development. Glutamic acid gives no measurable ammonia if incubated together with homogenates under the same conditions.

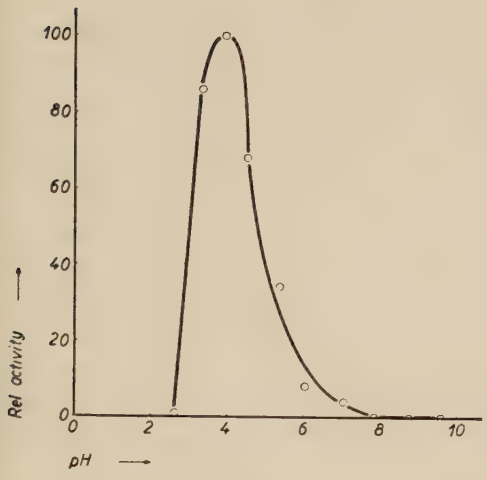

Fig. 14.

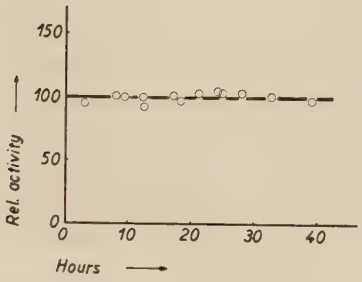

Fig. 15.

Fig. 14. Activity-pH curve of cathepsin, attacking denaturated hemoglobin. Material: Gastrulae of *Psammechinus miliaris*. Buffer: Veronal-acetate. No activator added.
Fig. 15. Relative catheptic activity in different developmental stages of *Psammechinus miliaris*. Substrate: Denaturated hemoglobin, no activator. Rate of development see fig. 6.

Proteolytic activity with denaturated hemoglobin as the substrate

Denaturated hemoglobin was used as substrate for studying the occurrence of catheptic activity. The method of Anson (3) was used. Homogenates of KCl washed eggs were prepared by swelling the eggs in ice cold distilled water, freezing, and vibration. The homogenates were stored for 1–3 days before use. The reaction mixtures were buffered by veronal-acetate buffers. No activator was added. The pH activity curve of homogenate of *Psammechinus* gastrulae shows an optimum at about 4 (fig. 14), and very low activity at neutrality. The enzymatic activity of homogenates from early cleavage stages is the same as that of more advanced stages up to pluteus stage (fig. 15). Li treated material has not been studied.

Cathepsin II

Fruton, Irving, and Bergmann (34) have defined cathepsin II as an enzyme that hydrolyzes *l*-benzoylarginine amide when an activator such as cysteine has been added. The racemic substrate was synthesized according to the description given by Bergmann, Fruton, and Pollok (11). The determinations of the enzymatic activity were made essentially according to Schultz (110). Cysteine was always added and the reaction mixtures were buffered by

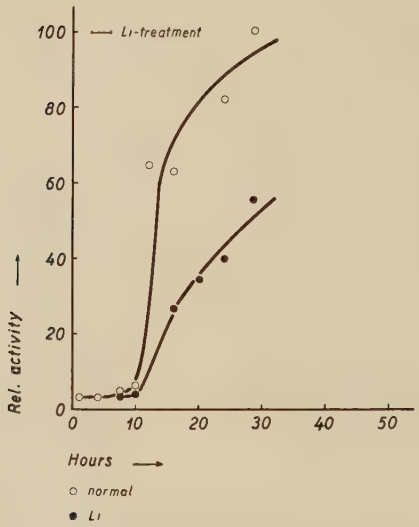

Fig. 16. Relative cathepsin II activity in different normal and Li treated developmental stages of *Psammechinus miliaris*. Primary mesenchyme at 11 hours during normal development, cf. fig. 6. Li treatment (9 parts 0.41 M LiCl to 91 parts sea water, cf. fig. 6 where the experiment was performed with animals from greater depth and higher salinity) gave rise to a rather weak vegetalization with only few exogastrulae.

veronal-acetate, phosphate or citrate. In preliminary experiments the homogenates from KCl-washed eggs, stored in a refrigerator box for one to three days, were diluted with isotonic KCl or distilled water. The reaction mixtures were incubated aerobically at room temperature or at 25° C for two to four hours. The ammonia production had a constant rate during this time.

During the course of the experiments it was found that the activity sometimes disappeared or was reduced. The pH optimum of the enzyme also varied somewhat in different experiments. These irregularities were not completely avoided by performing the assays on fresh samples. Since cathepsin II has been reported to show an increased activity anaerobically, possibly due to the formation of inhibitors in the presence of oxygen (63) further experiments were performed under strictly anaerobic conditions. In this case the KCl-washed fresh eggs were allowed to swell for 5 minutes in an ice bath in 0.05 M phosphate buffer at pH 7.0. The air had previously been extracted from the buffer. The tube with the eggs was then immersed in a mixture of NaCl and ice for two minutes with occasional shaking and was then vibrated for 90 seconds on the vibrator. During the whole procedure a current of hydrogen was passed through the sample. The homogenates

were then immediately transferred to a Thunberg tube with a buffer-substrate-cysteine mixture from which the air had been extracted previously. The Thunberg tube was then immediately evacuated and immersed in a water bath at 25° C and incubated for one hour. In this way the irregularities could be partly avoided and the activity was higher than in samples which had been run aerobically.

The activity of cathepsin II is very weak, or entirely missing up to mesenchymeblastula stage after which a strong rise occurs. This has been demonstrated for *Paracentrotus lividus, Sphœrechinus granularis, Strongylocentrotus drœbachiensis, Echinus esculentus,* and *Psammechinus miliaris.* Li treatment during cleavage stages of *Psammechinus miliaris* checks the subsequent rise in activity (fig. 16). Preliminary experiments indicate the same Li effects in *Paracentrotus* and *Sphaerechinus.*

Adenosine deaminase

Adenosine deaminase was determined by incubating one ml egg homogenate and 0.8 ml 0.2 M phosphate buffer and 0.2 ml 0.1 M adenosine. The substrate solution had to be heated immediately before use in order to dissolve all the adenosine crystals. The reaction mixture was incubated for 30 to 180 minutes at 22° C. The ammonia liberated was distilled by the Conway technique. The pH optimum of the enzymatic activity in 48 hour old *Psammechinus* plutei lies at about 6.0 (fig. 17). The adenosine deaminase activity in different developmental stages of the same species was determined at this pH. The homogenates were made differently in different experiments, i.e. the KCl washed eggs were swelled in distilled water or 0.05 M phosphate buffer of pH 7.0 for 5 minutes, the samples being kept in an ice-bath. The samples were then frozen in a NaCl-ice mixture, vibrated, frozen again and stored in the frozen state for 1 to 3 days before assay of the activity. This treatment does not seem to alter the activity significantly. The adenosine deaminase activity is constant throughout development both in normal and Li treated material (fig. 18).

Guanosine was used as the substrate in the same way as adenosine. 58 hour old plutei of *Psammechinus* had only weak activity at the same pH optimum as that of adenosine deaminase. The activity was about one fifth that of adenosine deaminase at pH 6.1. Since the purity of the commercial preparation of guanosine has not been checked the possibility cannot be excluded that the activity is due to contamination of the preparation by adenosine.

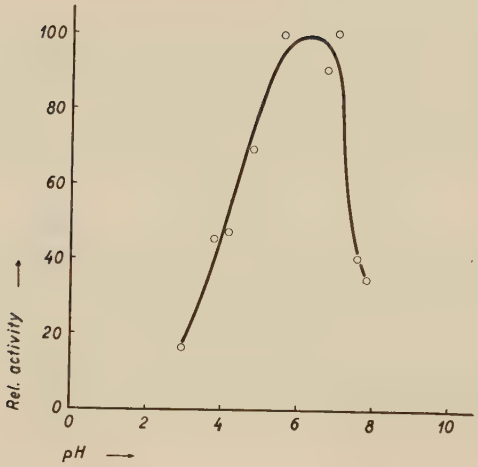

Fig. 17.

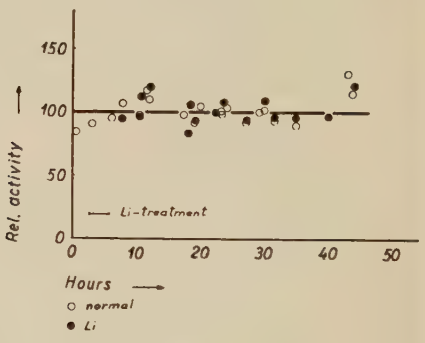

Fig. 18.

Fig. 17. Activity-pH curve of adenosine deaminase from plutei of *Psammechinus miliaris*. Buffer 0.04 M citrate below pH 5.6, 0.09 M phosphate above pH 5.6.
Fig. 18. Relative adenosine deaminase activity in different normal developmental stages of *Psammechinus miliaris*. Li treatment and rate of development see fig. 6.

Phenylsulphatase

Lindahl (73), who first demonstrated the presence of a phenolsulphatase in the sea urchin egg, determined the activity by measuring manometrically the CO_2 evolution from $CaCO_3$ by the acid liberated from phenylsulphate. No quantitative comparisons between the activity of different stages were recorded. Activity was, however, reported to be present in several different stages. The present assay of phenolsulphatase activity was performed according to the colorimetric method of Huggins and Smith (60). The substrate potassium-p-nitrophenylsulphate was prepared according to Huggins and Smith's adaptation of the method of Burkhardt and Lapworth. Veronal-acetate buffer was used and the reaction mixture was incubated for 3 to 15 minutes at 30° C. p-nitrophenol was liberated from the colorless substrate by the sulphatase in whole homogenates diluted with distilled water. The yellow color formed after addition of alkali was determined in the Klett-Summerson photoelectric colorimeter with a 420 mμ filter.

The pH optimum of the enzyme is about 6.0 (fig. 19) which is in good agreement with the pH optimum of 6.12 recorded by Huggins and Smith (60), using acetate buffer. Under the conditions used there was not, however, an exact linear relation between the rate of hydrolysis and enzymatic concentration.

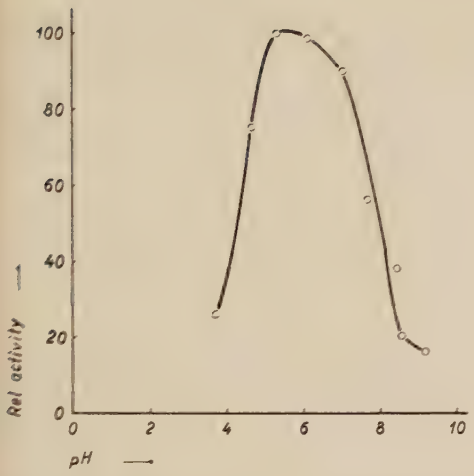

Fig. 19.

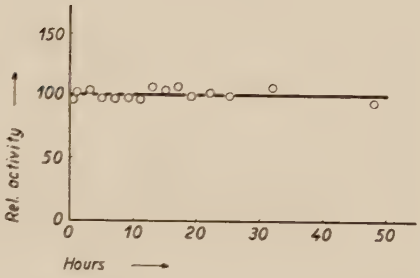

Fig. 20.

Fig. 19. Activity-pH curve of phenylsulphatase in gastrulae of *Paracentrotus lividus*. Buffer: Veronal-acetate.

Fig. 20. Relative phenylsulphatase activity in different developmental stages of *Paracentrotus lividus*. Rate of development see fig. 2.

Nor was there a straight line relationship between activity and periods of incubation. However, in the range of concentration in which the activity of different developmental stages were compared there is an approximately linear relation. There is thus no doubt that the phenolsulphatase activity is at a constant level throughout development of *Paracentrotus lividus* (fig. 20).

Ammonia content of homogenates

On assaying enzymes which split ammonia from their substrates the blanks contain significant amounts of ammonia, varying with the developmental stage of the homogenized larvae (fig. 21). The amounts are low and about the same in homogenates from different stages before the mesenchymeblastula, but become greater after that stage. Li treatment during cleavage stages slightly retards this ammonia formation. A pronounced retardation is, however, caused by a strong Li treatment which leads to the development of thickwalled, cell-filled blastulae, which die after 24 hours. The origin of the ammonia is not known. Part of it may be derived from amino acids which begin to be metabolized in the mesenchymeblastula. Calculations of the amount of protein oxidized during different periods of development, on the basis of the ammonia present in fresh homogenates, must be very haz-

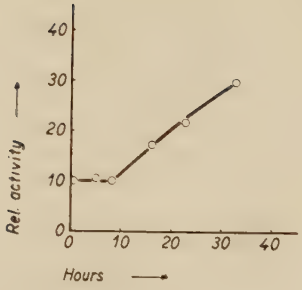

Fig. 21.

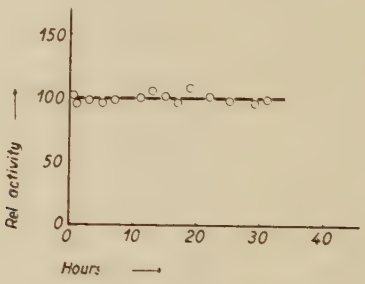

Fig. 22.

Fig. 21. Relative concentration of ammonia of homogenates from *Psammechinus miliaris* incubated in veronal-acetate buffer at 24° C for 2 hours at pH 8.7. Rate of development cf. fig. 6.

Fig. 22. Relative content of total N in different developmental stages of *Paracentrotus lividus*. Rate of development see fig. 6.

ardous, cf. (61). The ammonia liberated is probably consumed continuously for the synthesis of amino acids and amides, cf. (39, 40). In this connection it should be mentioned that the eggs do not loose any nitrogen, at least not before the early pluteus stage. This can be concluded from Kjeldahl nitrogen determinations on samples taken by the technique of sampling described above (fig. 22).

DISCUSSION

During the cleavage stages of the sea urchin egg, respiration increases according to an s-shaped curve (74, 15). Li treatment checks the further rise of respiratory intensity, but not the actual level, and the effect is reversible (73). Furthermore there is a close correspondence between the period of respiratory rise and the period during which Li causes vegetalization (75).

In spite of this important correlation, animal and vegetal halves have the same respiratory intensity, and their respiration is affected to the same extent by Li and some other agents (79). Furthermore checking of respiration by glyceraldehyde gives no typical vegetalization (74), cf. however (76).

Addition of an excess of K to the sea water counteracts the morphological effects of Li (103) as well as the effects upon respiration (73). Lindahl (73) suggests that Li possibly displaces K from certain groups of an enzyme. It has also been demonstrated by Ranzi *et al.* and others that the physical state of certain proteins in KCl solutions is changed by the addition of Li (100, cf. 37). According to a recent observation (66) K is necessary for phosphate transfer from 2-phospho-pyruvate to ADP in a partially purified rabbit

muscle system. This strongly suggests that Li, competing with K, may cause an abnormality in the phosphate metabolism. This has actually been demonstrated (80) in the case of yeasts, where addition of Li causes an accumulation of inorganic pyrophosphate. The existence of an enzyme which splits inorganic pyrophosphate from ATP is interesting in this connection (121).

One could assume, cf. (8, 64), that energy made available by oxidation of e.g. carbohydrates is accumulated in phosphate bonds, which mediate the energy to various synthetic reactions of importance to the processes of determination and differentiation. An ubiquitous compound with high energy bonds is ATP. This participates in a great variety of biological processes, the importance of which for embryonic processes has been demonstrated, cf. below. Thus the enzymatic background for synthesis and splitting of the energy rich phosphate bonds of ATP presents an important problem in developmental physiology. ATP splitting enzymes have been demonstrated in a wide variety of adult (see e.g. 27, 47, 85, 93) and embryonic tissues, cf. below.

The firm association between myosin and apyrase activity in muscle led Barth and Jaeger (8) to the suggestion that there might be a firm association between apyrases and other proteins or protein complexes as well. The energy from the ATP would be utilized for the building up of new proteins or protein complexes which are assumed to play an important role in embryonic development. It should also be important for the embryonic movements.

The results on the sea urchin egg show that the apyrase activity is constant during the period of primary determination of the germ layers, in spite of the increased respiration during that time. The activity does not rise until the onset of visible differentiation, i.e. at the mesenchymeblastula stage. This result is in very good accordance with a number of studies made by other authors on various kinds of material. Thus Barth and Jaeger (8) found at least three different apyrase protein fractions in the developing frog egg. They present evidence in favour of the opinion that a single apyrase is closely associated with different proteins. All these fractions have a low activity during early cleavage stages and increase in activity during gastrulation. The shapes of the curves are different from each other. The same authors found that the ATP content of the germs remained unchanged from cleavage until early neurula stage. In anaerobiosis the breakdown of ATP was small during the cleavage stages, where development proceeded, whereas the breakdown was much stronger in the gastrula stage where development ceased. The same held for hybrid gastrulae, which had a decreased capacity for maintaining ATP in the phosphorylated state (9). In the chick

embryo (94) the apyrase activity is comparatively low up to the sixth day, at least from about the third day. After this time the activity shows a rise which can be correlated with a rise in the N content of the embryo, i.e. with an increasing synthetic activity. In fetal guinea pig brain cortex apyrase activity is at a constant level up to the fortysecond day of gestation after which an increase occurs. Much the same seems to hold for the embryonic liver in this species (30). In brain and liver of the new born rat there is an abrupt increase in apyrase during a period of greatest development (99). In the composite ascidian Symplegnia the regeneration of zooids from the stolons is associated with a strong rise in apyrase activity (64). Thus it seems to be generally true that apyrases increase in activity parallel to growth and histodifferentiation. At this time ATP must play an important role. It is then easy to imagine that Li can interfere in various kinds of embryonic processes (37).

The importance of particulate elements in embryogenesis has been repeatedly stressed see e.g. (7, 37) and the energy supply through ATP for the replication of these elements has been suggested by Barth and Jaeger (8). It is thus interesting that apyrase activity in the chick embryo is to a great extent localized in the granules and apparently becomes more concentrated in these granules as development proceeds (117). In spite of the uncertain origin of the granules one may assume that they represent active enzyme units of cell structure (117). These granules are complex enzyme units since cytochrome oxidase and related enzymes are also located in them (117). In fact the present results from the sea urchin egg suggest that a whole group of enzymes increase in about the same manner, and there is much evidence that most of them are bound to granules in the cells, i.e. to mitochondria. Thus apyrase, succinodehydrogenase, malic dehydrogenase, glutaminase, and cathepsin II have a comparatively low or no activity up to about the mesenchyme blastula stage, after which their activity increases. Apyrase, glutaminase, cathepsin II, and according to preliminary experiments also succinodehydrogenase are checked with respect to their rise in activity by previous Li treatment.

Of these enzymes succinodehydrogenase and malic dehydrogenase are typical mitochondrial enzymes (59). Cathepsin II is probably bound to cytoplasmic granulae (110).

Krebs (68), Archibald (4), and Greenstein and Leuthardt (36) have come to the conclusion that there are two different types of glutaminase. The pH optimum of the present glutaminase indicates that it could be classified as a glutaminase I. This enzyme is localized in the insoluble portion of liver

homogenates (29), whereas glutaminase II is soluble. A great part of the glutaminase from dog kidney adheres tenaciously to cellular components insoluble in ordinary extractives (4). One can thus assume that the glutaminase activity in the sea urchin egg is also to a large extent bound to the insoluble portion of the cytoplasm, e.g. to mitochondria.

Other authors have also reported an increase of some mitochondrial enzymes parallel to an increase in differentiation, mass and functional development in various material. Thus in the chick embryo cytochrome oxidase activity begins to increase at about the same time as that of apyrase (1), and hence parallels an increase in N content of the embryo. In the cerebral cortex of the fetal pig succinodehydrogenase and succinoxidase activity rise between two critical periods of cytological development. During the latter stage the neuron attains a mature appearance. The rise in succinoxidase activity seems to depend upon a rise in cytochrome C content (31, 32). Succinodehydrogenase in the whole brain and liver of the rat rises at about the time of birth and is parallelled by the increase in wet weight and apyrase mentioned above (99). The later rise in enzymatic activities in rat brain as compared with pig and guinea pig cerebral cortex seems to be correlated with the lower degree of maturity at birth in the rat (30, 32).

In some of the cases cited above pure differentiation may be accompanied by growth migration and preparation for special functional activity. We have, however, good chemical evidence for suggesting that histodifferentiation in the sea urchin embryo and elsewhere is accompanied by the development of units of associated enzymes, i.e. of mitochondria. Preliminary cytological studies on the frog egg support this conclusion, demonstrating that mitochondria begin to appear in those areas where morphogenesis is earliest and strongest (71). Likewise preliminary studies with the phase contrast microscope and vital staining by the present authors show that mitochondria begin to increase in number at those stages where the enzymatic rise in activity begins.

The coordination between the enzymes in the mitochondria is evident from the sea urchin egg. The carbohydrates of the yolk granulae (91, 92) may be utilized for energy production by means of succinodehydrogenase, malic dehydrogenase, and other enzymes. The energy released is stored or at least transferred to phosphate bonds which is in good accordance with the fact that the ATP-turnover seems to increase at about the mesenchyme-blastula stage (82). The phosphate bond energy may be transferred by the apyrase to various synthetic reactions, e.g. to glutamine synthesis which is known to consume ATP (28, 18, 114, 115, 116).

The developmental changes in glutamine content and glutaminase activity suggest (40) an increased turnover rate of glutamine at differentiation and a slower increase after Li treatment. The importance of glutaminase I for protein synthesis is strongly suggested by the present result, by the strong rise in glutaminase I activity during cancer formation in rat and mouse liver (35), and also by the fact that anterior pituitary growth hormone preparations increase the kidney glutaminase of hypophysectomized rats (10). The possible role of glutamine in peptide synthesis is suggested by the energetic similarity between the amide and true peptide linkages (115) and is visualized by Waelsch *et al.* (119). The quantitative developmental changes of the peptide containing extracts from sea urchin eggs (40) fit well with such a hypothesis.

During synthesis of proteins the mitochondrial cathepsin II may play an important role (110). The final products may form new mitochondrial material as well as specific tissue proteins. The appearance of new antigens in connection with differentiation may be an expression of this synthetic activity (98).

The ectodermal development may be characterized by a more intensive mitochondrial activity than the vegetal one. This may be in good accordance with the observation of Child, cf. (77), that Janus-green may cause vegetalization. Janus-green is frequently used as a specific vital stain for mitochondria. Leuthardt *et al.* (72) have demonstrated that Janus-green interferes with citrulline synthesis in rat liver mitochondria. It is probable that it may also interfere with the well organized pattern of mitochondrial enzymes which probably cooperate during the synthesis of proteins.

After the exponential rise in respiratory intensity (74, 15) there is a constant activity from e.g. 6 to 9 hours of development. After that time respiration begins to increase abruptly in connection with the preparation for the migration of the primary mesenchyme cells into the blastocoel. Lindahl (74) suggests that this is an indication that new metabolic processes start in the egg. Our enzymatic studies are in good agreement with this opinion. More precisely the respiratory rise should reflect the increased density of the mitochondrial population. This could be expected to correspond to a rather harmonious increase in all respiratory enzymes, which are probably situated in the mitochondria, cf. (111, 96).

Some enzymes show changes in activity which follow a different general course from that of the enzymes studied in the present work. For cholinesterase there seems to be a close correlation between the development of ciliary and muscular movements and cholinesterase synthesis (5) cf. (14).

It has been shown above that acid phosphatase, phosphatases attacking inorganic pyro- and hexametaphosphate, aldolase, phenylsulphatase, adenosine deaminase, and a protease do not change in activity during development. One could assume that this might be the case with a series of enzymes which are dissolved in the non-mitochondrial fraction of the cytoplasm and not produced by mitochondria.

The carbohydrate metabolism of the sea urchin egg has been subject to much interest, cf. (83). Runnström (107) found that monoiodacetate does not inhibit the increased respiration after fertilization and concluded that the carbohydrate breakdown does not follow the pathway of glycolysis as it occurs in e.g. muscle. Lindberg (81) suggested that the carbohydrate breakdown in unfertilized and newly fertilized eggs proceeds from hexose-6-phosphate to phosphogluconic acid. Further evidences for the suggested pathway of carbohydrate breakdown have come from experiments on the effect of glucose, hexosemonophosphate, and hexosediphosphate on respiration of homogenates with and without addition of monoiodacetic acid (83). Lindberg and Ernster (83) conclude that the aldolase reaction is of little importance in the oxidation of carbohydrates in homogenized sea urchin eggs. In spite of the abnormal pH curve we must however draw the conclusion from the present experiments that aldolase is present in the eggs, possibly together with phosphotriose isomerase. Aldolase in the sea urchin egg may play a minor role in addition to the pathway suggested by Lindberg. It may also function mainly in later development. Furthermore it may have its main importance for synthesis of hexoses from trioses. It is important that, according to Sibley *et al.* (112), the activity of aldolase in different tissues is quite high, indicating a considerable reserve of aldolase above that required to account for known rates of carbohydrate metabolism. As to the intracellular localization of aldolase it appears to be diffusely distributed and to be present in both nuclei and cytoplasm (26, 2). Thus there seems to be no reason to assume that it should be concentrated in mitochondria. In good accordance with this the activity is constant throughout development and does not increase as do typical mitochondrial enzymes.

Herbst (45, 46) demonstrated that sulphate begins to be needed by the embryo at an advanced blastula stage. Lindahl (73) suggested that the sulphate is needed for detoxification of waste products of aromatic amino acids, locally produced in parts of the eggs with high vegetal activity. He demonstrated the existence of a phenol-sulphatase which could catalyze the esterification of the aromatic products in the egg. A sulphatase of a different specificity has been found by other authors in echinoderms (113).

Since the specific substrate for the sulphatase is likely to appear at about the mesenchyme blastula stage, cf. (40), and enzyme adaptation could possibly occur, the quantitative assay of the enzyme was undertaken. The high activity of this enzyme in rat neoplasms (60) also emphasizes the embryological role of this enzyme, and so does the suggested relation between pigment formation and sulphate metabolism in the sea urchin egg (73). The activity of phenylsulphatase is evidently constant throughout development according to the present studies. The course of the curve has not indicated the necessity for studies of Li larvae. Since phenylsulphatase of plant as well as of animal origin is not tightly bound to the solid portion of the cell (33) one can assume that it is not a mitochondrial enzyme but dissolved in the nonparticular cytoplasmic fraction.

The enzymatic activity upon denaturated hemoglobin at an acid pH, as opposed to the activity of cathepsin II, does not evidently change during development of the *Psammechinus* egg. The same result has been reported for a peptidase, attacking alanyl-glycine at a slightly alkaline reaction (49, 48, 84). In the case of this enzymatic activity it has been demonstrated by Holter and Lindahl (49) that it is not selectively concentrated in certain parts of the egg and larvae. Furthermore, studies of Holter (48) on fragments of eggs stratified by centrifugation show that there is no relation between peptidase activity and any formed cytoplasmic constituents. The peptidase activity follows the distribution of the hyaline ground cytoplasm. Holter concludes that it is diffusely distributed throughout the cytoplasmic matrix. Furthermore, Holter states that if mitochondria should secrete peptidase, this would occur chiefly in the immature ovum. On the basis of his own experiments and the findings of Shapiro (111) and Navez and Harvey (96) he suggests that respiratory and hydrolytic enzymes, at least peptidases, should have an essentially different localization. This is in very good agreement with the interpretation of our own results.

It has been suggested that the cholinesterase activity begins to rise later in the intestine than in the rest of the larva (5). The rise in activity can be correlated with the appearance of coordinated contractile activity in the intestine. One might suggest that the difference in the activity curves of the normal and Li treated stages also in other cases could be an expression of such differences in rate of development between different parts of the egg. When advanced normal and Li treated stages have been compared the differences are, however, still present at 40 hours or later, cf. glutaminase and apyrase. After this time the normal larvae are fully developed plutei. Also the Li treated eggs have attained their final appearance with a thin small ciliated ectoderm

vesicle and an intestine which is divided into three parts. It may, however, be difficult to state at which time strongly vegetalized larvae should be considered to be "fully differentiated." Owing to the difficulties in comparing Li treated stages with definite stages in normal development we must limit our conclusions to the statement that Li vegetalization lowers the rate of enzyme synthesis as compared with normal larvae. It is, however, very tempting to suggest that the more efficient mechanism of synthesis in normal larvae compared with Li larvae at the same time after fertilization indicates that the mechanism for synthesis develops more strongly in the ectodermal parts than in the mesentodermal parts of normal larvae. It appears at least that when the synthetic mechanism is checked by Li treatment development follows another pathway which is characterized by lower synthetic activity in its earlier phases.

Li added during the period of primary determination of the germ layers does not cause any immediate effects on the enzyme pattern as far as the present studies have shown. Effects do not appear until the mesenchyme-blastula stage at a time when the larvae have already been back in normal sea water for two or three hours. The normal developmental changes and the effects of Li may, as suggested above, reflect the development of mitochondria. Up to the mesenchymeblastula stage the mitochondrial population should be weak. Li treatment during early developmental stages evidently determines the growth rate of the mitochondrial populations in later stages.

Chantrenne (19) has demonstrated that adult mouse liver granulae, sedimentable by high speed centrifugation, cannot be separated only into two main groups, big granulae and microsomes, as suggested by Claude (20). It is rather a continuous variation both in sizes and chemical composition. The greater the particles, the smaller their ribonucleic acid content and the greater their content of alkaline phosphatase, adenylpyrophosphatase, and phospholipids. Chantrenne suggests that the granulae of different sizes represent different developmental stages of lipoprotein complexes which grow and elaborate enzymes around centers rich in ribonucleic acid. Jeener (65) has demonstrated that the granulae of various sizes can be fragmented into particles of various sizes and chemical composition by the treatment with Edsall solution. The smaller the size of the fragments, the higher their content of ribonucleic acid and the lower their enzymatic activity. The smaller the granulae, the higher is the proportion of small nucleic acid rich fragments at fragmentation. On the basis of these observations and after discussion of the growth of certain viruses, Jeener suggests that small nucleic acid rich particles gradually aggregate and that the interior of these

aggregates is the site of enzyme production. This enzyme production is paralleled by a disappearance of ribonucleic acid in the interior of each aggregate while new nucleic acid rich particles are continually added to the periphery. Mitochondria should constitute the end stage of this developmental process.

This gradual elaboration of bigger granulae may play an important role in embryonic development, as suggested by Brachet and Chantrenne (16). These authors support their view on the basis of the observation of a progressive combination of "free" nucleic acid with large granulae as embryonic development proceeds. The appearance of fuchsinophil small granulae in the early frog egg development (67) is a cytological indication of such a stepwise elaboration of mitochondria. Furthermore the experiments of Harvey (42) suggest that mitochondria may arise from non-mitochondrial precursors, since mitochondria free egg fragments, produced by centrifugal fragmentation, may develop slowly into rather normal plutei. The mitochondria are replaced in the plutei, but not during early cleavage stages. This is in good agreement with our own cytological and chemical studies. Certain small particles can be removed from the clear cytoplasm with still stronger centrifugation of the fragments. These may represent intermediate stages in the elaboration of mitochondria. The chromidia of the sea urchin egg are very minute nucleic acid rich particles, not displaceable from the ground cytoplasm (90). These elements may constitute still earlier stages in the mitochondrial development.

The period of primary determination of the germ layers could thus be characterized by a stepwise elaboration of germs for mitochondria. The beginning enzyme synthesis in the interior of these germs seems to start at a stage where the nuclear activity becomes intensified, cf. (37). The number of germs at this time may determine the intensity of enzyme synthesis. There seems to be several possibilities for Li to interfere with the synthesis of such germs, since Li treatment interferes with the phosphate metabolism as well as with the physical state of proteins. The number of germs which begin to produce enzymes at the critical time is probably lower after Li treatment in an early stage than in normal development.

According to recent studies (50), microsomes appear to be rich in DPN-cytochrome C reductase. A growth of the microsome population should thus be manifested e.g. by a rise in DPN-cytochrome C reductase activity. One may tentatively suggest that this is the cause of the S-shaped rise in respiration during cleavage stages, which can be checked in its further course by the addition of Li.

It will, however, be difficult to understand the qualitative differences be-

tween the animal and vegetal development on the basis of quantitative differences alone. For example, the development of relatively uncomplicated structures such as the animal stereocilia and the vegetal pigment, which accumulate abundantly in animalized and vegetalized larvae respectively, are likely to need a qualitatively different mechanism for their synthesis.

One of the fundaments of the double gradient conception of Runnström, is the interaction between animal and vegetal parts (52, 73). The vegetal parts must give off substances which, diffusing in the animal direction, check the development of the ciliary tuft, etc. They may even cause an isolated animal half to develop ciliary bands, stomodaeum and gastrulate. Such compounds may be intermediates in the protein synthesis brought about by the collaboration of the mitochondrial mechanism and the enzymes dissolved in the surrounding cytoplasm. Runnström has drawn our attention to experiments by Davis (23) who has shown that *Escherichia coli*, blocked by mutation at a certain stage in isoleucine synthesis, accumulates an intermediate. This compound competitively inhibits an enzyme essential for the synthesis of the structurally related compound valine. Analogously, intermediates in the protein synthesis accumulated in the vegetal parts of the egg, may diffuse in animal direction and interfere with the processes of synthesis of animal stereocilia, etc. Various compounds, such as amino acids may have the same effect during the development of animal fragments (55, 56, 57, 58). The red pigment is one example of vegetal accumulation products.

Experiments on the effect of agents extractable by 70 per cent alcohol upon the growth of *Lactobacillus casei*, cultivated on a synthetic basal medium, may be of interest in this connection (41). These have shown that Li treated larvae contain more growth-stimulating agents during pregastrular stages than normal eggs of the same age. Thus vegetal parts may contain more of these compounds than animal parts. The nature of these compounds is still unknown. The experiments were, however, carried out in such a manner that the presence of some peptides would stimulate growth. It is known that certain peptides stimulate growth of *Lactobacillus casei* (120). It is tempting to suggest that the growth stimulating principle of the sea urchin egg extracts are intermediates in the protein metabolism.

SUMMARY

The relative activity of a number of different enzymes has been determined in different developmental stages of normal and Li treated sea urchin eggs.

Some enzymes show a constant activity throughout development, whereas the activity of other enzymes is constant up to mesenchymeblastula stage and then increases. A short review of enzymatic changes in other embryonic material is presented. This demonstrated a striking agreement with the sea urchin egg in essential points.

It has been suggested that the enzymes which rise in activity at mesenchymeblastula stage are localized in mitochondria, whereas enzymes with constant activity are non-mitochondrial.

Li treatment during cleavage stages, which causes vegetalization, inhibits the rise in activity of apyrase, cathepsin II, glutaminase, and according to preliminary experiments also of succinodehydrogenase.

The authors conclude that Li treatment during cleavage stages interferes with the later development of mitochondria, possibly by inhibiting the development of their precursors.

According to these results, the ectodermal development is characterized by a more important mitochondrial activity, i.e. a more elaborate mechanism of synthesis, than the mesentodermal development. This is at least the case during earlier stages of development.

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REFERENCES

1. Albaum, H. G., and Worley, L. G., *J. Biol. Chem.*, **144**, 697 (1942).
2. Allen, R. J. L., and Bourne, G. J., *J. Exptl. Biol.*, **20**, 61 (1943).
3. Anson, M. L., *J. Gen. Physiol.*, **20**, 565 (1936–1937).
4. Archibald, R. M., *J. Biol. Chem.*, **154**, 657 (1944).
5. Augustinsson, K.-B., and Gustafson, T., *J. Cellular Comp. Physiol.*, **34**, 311 (1949).
6. Bailey, K., *Biochem. J.*, **45**, 479 (1949).
7. Barth, L. G., Colloid Chemistry. V. Ed. Alexander, J., p. 851, 1944.
8. Barth, L. G., and Jaeger, L., *J. Cellular Comp. Physiol.*, **30**, 111 (1947).
9. —— *Physiol. Zoöl.*, **20**, 133 (1948).
10. Bartlett, P. D., and Gaebler, O. H., *J. Biol. Chem.*, **181**, 523 (1949).
11. Bergman, M., Fruton, J. S., and Pollok, H., *ibid.*, **127**, 643 (1939).
12. Binkley, F., Shank, R. E., and Hoagland, Ch. L., *ibid.*, **156**, 253 (1944).
13. Biochemical Preparations, I. Ed. Carter, H. E., New York, p. 44, 1949.
14. Boell, E. J., *Ann. N. Y. Acad. Sci.*, **49**, 773 (1947).
15. Borei, H., *Biol. Bull.*, **95**, 124 (1948).
16. Brachet, J., and Chantrenne, H., *Acta Biol. Belg.*, **2**, 451 (1944).

17. Briggs, A. B., *J. Biol. Chem.*, **53**, 13 (1922).
18. Bujard, E., and Leuthardt, F., *Helv. Physiol. et Pharmacol. Acta*, **5**, C 39 (1947).
19. Chantrenne, H., *Biochim. et Biophys. Acta*, **1**, 437 (1947).
20. Claude, A., *J. Exptl. Med.*, **84**, 51, 61 (1946)..
21. Connors, W. M., and Scheer, B. T., *J. Cellular Comp. Physiol.*, **30**, 271 (1947).
22. Conway, E. J., Microdiffusion Analysis and Volumetric Error. London, 1947.
23. Davis, B. D., *J. Clin. Invest.*, **29**, 808 (1950).
24. Dounce, A. L., The Enzymes, Chemistry and Mechanism of Action. I. Ed. Sumner, J. B., and Myrbäck, K., p. 187, 1950.
25. Dounce, A. L., Barnett, S. R., and Beyer, G. T., *J. Biol. Chem.*, **185**, 769 (1950).
26. Dounce, A. L., and Beyer, G. T., *J. Biol. Chem.*, **174**, 159 (1948).
27. Du Bois, K. P., and Potter, V. R., *J. Biol. Chem.*, **150**, 185 (1943).
28. Elliot, W. H., *Nature*, **161**, 128 (1948).
29. Errera, M., *J. Biol. Chem.*, **178**, 483 (1949).
30. Flexner, J. B., and Flexner, L. B., *J. Cellular Comp. Physiol.*, **31**, 311 (1948).
31. Flexner, J. B., Flexner, L. B., and Straus, Jr., W. L., *ibid.*, **18**, 355 (1941).
32. Flexner, L. B., and Flexner, J. B., *ibid.*, **27**, 35 (1946).
33. Fromageot, C., The Enzymes, Chemistry and Mechanism of Action. I. Ed. Sumner, J. B., and Myrbäck, K., p. 517, 1950.
34. Fruton, J. S., Irving, G. W., Jr., and Bergmann, M., *J. Biol. Chem.*, **138**, 249 (1941).
35. Greenstein, J. P., Fodor, P. J., and Leuthardt, F. M., *J. Natl. Cancer Inst.*, **10**, 271 (1949).
36. Greenstein, J. P., and Leuthardt, F. M., *Arch. Biochem.*, **17**, 105 (1948).
37. Gustafson, T., *Rev. Suisse Zool.*, **57**, suppl. 1, 77 (1950).
38. Gustafson, T., and Hasselberg, I., *Exptl. Cell. Res.*, **1**, 371 (1950).
39. Gustafson, T., and Hjelte, M.-B., *Arkiv Kemi*, **2**, 363 (1950).
40. —— *Exptl. Cell Res.*, **2**, 474 (1951).
41. —— Unpublished.
42. Harvey, E. B., *J. Exptl. Zoöl.*, **102**, 253 (1946).
43. Herbst C., *Z. Wiss. Zoöl.*, **55**, 446 (1892).
44. —— *Mitt. Zool. Stat. Neapel*, **11**, 136 (1893).
45. —— *Arch. Entwicklungsmech. Organ*, **5** (1897).
46. —— *ibid.*, **17** (1904).
47. Hermann, H., and Hartmann, W., *Proc. Soc. Exptl. Biol. Med.*, **68**, 346 (1948).
48. Holter, H., *J. Cellular Comp. Physiol.*, **8**, 179 (1936).
49. Holter, H., and Lindahl, P.-E., *Compt. Rend. Trav. Lab. Carlsberg. Sér. Chim.*, **23**, 249 (1940).
50. Hoogebom, G. E., *J. Biol. Chem.*, **177**, 847 (1949).
51. Hörstadius, S., *Acta Zoöl.*, **9**, 1 (1928).
52. —— *Pubbl. Staz. Zool. Napoli*, **14**, 251 (1935).
53. —— *Arch. Entwicklungsmech. Organ.*, **135**, 1 (1936).
54. —— *Biol. Revs.*, **14**, 132 (1939).
55. —— *Pubbl. Staz. Zool. Napoli, Suppl.*, **21**, 131 (1949).
56. Hörstadius, S., and Gustafson, T., *Zool. Bidr. Upsala*, **25**, 571 (1947).
57. —— *Symposia Soc. Exptl. Biol.*, **2**, 50 (1948).
58. —— Unpublished.
59. Huennekens, F. M., *Exptl. Cell Res.*, **2**, 115 (1951).
60. Huggins, C., and Smith, D. R., *J. Biol. Chem.*, **170**, 391 (1947).
61. Hutchens, J. O., Keltch, A. K , Krahl, M. E., and Clowes, G. H. A., *J. Gen. Physiol.*, **25**, 717 (1941).
62. Ingelman, B., The Enzymes, Chemistry and Mechanism of Action. I. Ed. Sumner, J. B., and Myrbäck, K., p. 511, 1950.
63. Irving, Jr., G. W., Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **144**, 161 (1942).
64. Jaeger, L., and Barth, L. G., *J. Cellular Comp. Physiol.*, **32**, 319 (1948).
65. Jeener, R., *Biochim. et Biophys. Acta*, **2**, 633 (1948).
66. Kachmar, J. F., and Boyer, P. D., *Federation Proc.*, **10**, 204 (1951).
67. Konopacki, M., *Bull. Histol. Appl. Physiol. et Path. et Tech. Microscop.*, **4**, 40 (1927).
68. Krebs, H. A., *Biochem. J.*, **29**, 1951 (1935).

69. Krugelis, E. J., *Biol. Bull.*, **93**, 209 (1947).
70. Kun, E., and Abood, L. G., *Science*, **109**, 144 (1949).
71. Lenicque, P., Unpublished.
72. Leuthardt, F., and Müller, A. F., *Experientia*, **4**, 478 (1948).
73. Lindahl, P.-E., *Acta Zoöl.*, **17**, 179 (1936).
74. —— *Z. Vergleich. Physiol.*, **27**, 233 (1939).
75. —— *Arch. Entwicklungsmech. Organ.*, **140**, 168 (1940).
76. —— *Naturwissenschaften*, **29**, 673 (1941).
77. —— *Protoplasma*, **36**, 558 (1942).
78. —— *Quart. Rev. Biol.*, **17** (1942).
79. Lindahl, P.-E., and Holter, H., *Compt. Rend. Trav. Lab. Carlsberg. Sér. Chim.*, **23**, 257 (1940).
80. Lindahl, P.-E., and Lindberg, O., *Nature*, **157**, 335 (1946).
81. Lindberg, O., *Arkiv Kemi, Mineral, Geol.*, **16** *A*, **15** (1943).
82. —— *Exptl. Cell Res.*, **1**, 105 (1950).
83. Lindberg, O., and Ernster, L., *Biochim. et Biophys. Acta*, **2**, 471 (1948).
84. Linderström-Lang, K., *Medd. fra Carlsberg. Lab.*, **19**, 1 (1933).
85. Lipschitz, R., and Frank, S., *J. Cellular Comp. Physiol.*, **33**, 27 (1949).
86. Mazia, D., Blumenthal, G., and Benson, E., *Biol. Bull.*, **95**, 250 (1948).
87. Meyerhof, O., and Beck, L. V., *J. Biol. Chem.*, **156**, 109 (1944).
88. Meyerhof, O., and Junowicz-Kocholaty, R., *J. Biol. Chem.*, **149**, 71 (1943).
89. Meyerhof, O., and Wilson, J. R., *Arch. Biochem.*, **21**, 1 (1949).
90. Monné, L., *Experientia*, **2**, 153 (1946).
91. Monné, L., and Slautterback, D. B., *Arkiv. Zool.*, **1**, 455 (1950).
92. —— *Exptl. Cell Res.*, **1**, 477 (1950).
93. Moog, F., *J. Exptl. Zoöl.*, **105**, 209 (1947).
94. Moog, F., and Steinbach, H. B., *J. Cellular Comp. Physiol.*, **25**, 133 (1945).
95. Naganna, B., and Menon, V. K. N., *J. Biol. Chem.*, **174**, 501 (1948).
96. Navez, A. E., and Harvey, E. B., *Biol. Bull.*, **69**, 342 (1935).
97. Oesper, P., and Meyerhof, O., *Arch. Biochem.*, **27**, 223 (1950).
98. Perlmann, P., and Gustafson, T., *Experientia*, **4**, 481 (1948).
99. Potter, V. R., Schneider, W. C., and Liebl, G. J., *Cancer Res.*, **5**, 21 (1945).
100. Ranzi, S., *Experientia*, **7**, 169 (1951).
101. Roche, J., The Enzymes, Chemistry and Mechanism of Action. I. Ed. Sumner, J. B. and Myrbäck, K., p. 473, 1950.
102. Runnström, J., *Arch. Entwicklungsmech. Organ*, **113** (1928).
103. —— *Acta Zool.*, **9**, 1 (1928).
104. —— *Arch. Entwicklungsmech. Organ.*, **117**, (1929).
105. —— *ibid.*, **124**, (1931).
106. —— *ibid.*, **129**, (1933).
107. —— *Biol. Bull.*, **69**, 351 (1935).
108. —— Lecture in Cambridge, 1949, unpublished.
109. Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, **149**, 217 (1943).
110. Schultz, J., *ibid.*, **178**, 451 (1949).
111. Shapiro, H., *J. Cellular Comp. Physiol.*, **6**, 101 (1935).
112. Sibley, J. A., and Lehninger, A. L., *J. Biol. Chem.*, **177**, 859 (1949).
113. Soda, T., and Egami, F., *J. Chem. Soc. Japan*, **54**, 1069 (1933).
114. Speck, J. F., *J. Biol. Chem.*, **168**, 403 (1947).
115. Speck, J. F., *ibid.*, **179**, 1387 (1949).
116. —— *ibid.*, **179**, 1405 (1949).
117. Steinbach, H. B., and Moog, F., *J. Cellular Comp. Physiol.*, **26**, 175 (1945).
118. Utter, M. F., and Werkman, C. H., *J. Bact.*, **42**, 665 (1941).
119. Waelsch, H., Borek, E., and Grossowicz, N., 54 c, Am. Chem. Soc. Proc., Sept. 1949.
120. Wolley, D. W., *J. Biol. Chem.*, **172**, 71 (1948).
121. Zeller, E. A., *Arch. Biochem.*, **28**, 138 (1950).
122. Zittle, Ch. A., The Enzymes, Chemistry and Mechanism of Action. I. Ed. Sumner, J. B., and Myrbäck, K., p. 222, 1951.

DIFFRACTION OF ELECTRONS BY STRUCTURES RESEMBLING MYELIN LAMELLAE

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The electron microscope images of thin layers isolated from the myelin sheath of osmium fixed vertebrate nerve fibres frequently demonstrate certain wavy dark bands (7), which are similar to the patterns described as extinction contours in split mica by Boersch (4), Heidenreich and Sturkey (10) and Heidenreich (9). Upon closer examination, "reflection images" of the type described by Heidenreich and Sturkey (10) and by Boersch (5) as "Nebenbilder," have been observed next to the transmitted images of these osmium fixed lamellae in extrafocal micrographs taken with the objective aperture removed. Since both of these effects are exhibited by crystalline materials only and represent crystal interference phenomena, it appeared possible that this type of lamellae might also yield characteristic electron diffraction patterns. Piles of lamellae thin enough for electron transmission experiments are generally only a few microns long, and methods must therefore be employed for obtaining electron diffraction patterns from restricted areas of the specimen in the electron microscope (1, 3, 6).

By making use of a fine condenser aperture in the device described by Boadway (2), installed in an RCA electron microscope which is fitted with a removable intermediate lens (11), electron diffraction patterns may be taken from small areas of a preparation in the normal object position and an electron micrograph of the corresponding field obtained immediately, *without removing the objective and projector pole pieces, breaking the vacuum or otherwise altering the alignment of the instrument.*

This arrangement is specially suitable for continuous parallel observation of the morphology of the irradiated specimen and correlation with its diffraction pattern. With it, electron diffraction patterns have been obtained from lamellar structures resembling myelin lamellae, isolated from rat and frog sciatic nerve fibres fixed with osmium, which will be described in this report. Since there is evidence that these structures are not true myelin lamellae but crystalline osmium products formed in the process of nerve fixation, they will be referred to here as osmium lamellae.

MATERIAL AND METHODS

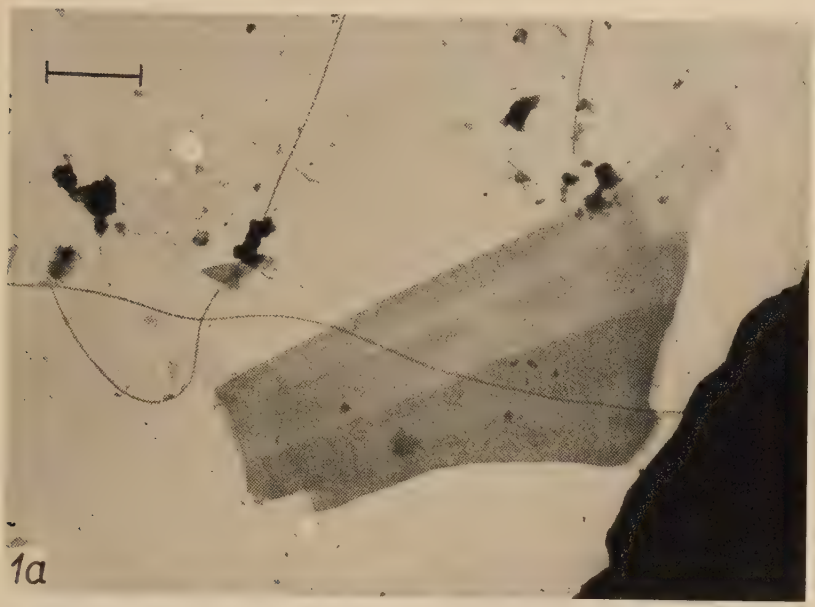
Sciatic and spinal motor root nerves from albino rats and bullfrogs were fixed in buffered (pH 7.08–7.20) solutions of 1 per cent osmium tetroxide or 10 per cent formalin, for 7 to 24 hours. The lamellar structures were isolated by gentle dissociation of single nerve fibres under the light microscope. Control specimens of myelin lamellae were obtained from thin longitudinal or tangential frozen sections of fixed nerve fibres (7). All preparations were mounted on collodion films, taking precautions to insure utmost cleanliness and avoid contamination. None of the collodion supporting films used exhibited the polycrystalline or single crystal structure reported in the literature (8).

The regular condenser aperture and screen of an RCA electron microscope, model EMU with a biased gun and an accelerating potential of 40 kV, was replaced by the device described by Boadway (2) for holding a thin platinum diaphragm (20 microns in thickness) with a hole 150 or 200 microns in diameter. A commercial removable intermediate lens supplied by RCA was used, and both the intermediate and the projector lens were provided with extra apertures of 5 mm and 1 mm diameter respectively. Instead of reducing the high voltage to 40 kV, the focal length of the intermediate lens can also be altered for diffraction work by increasing its coil current when using the standard 50 kV.

Once the fine aperture is properly aligned with the magnetic axis of the condenser (2), electron diffraction patterns may be taken of the specimen in the regular object position by focusing the beam to an area of an approximate cross section of 20–50 microns on the specimen film, and turning down the magnification controls (projection and intermediate lens) to the lowest setting. An electron diffraction pattern of the centered field is then observed on the viewing screen, which can be instantly substituted for the corresponding image of the object by switching back to higher magnifications. The whole preparation can thus be scanned for suitable lamellar structures lying single in isolated areas of the collodion film, and serial electron micrographs and electron diffraction patterns of the same specimen taken alternately. Appropriate fields were selected in which only one pile of lamellae could be responsible for the electron diffraction pattern recorded. Parallel controls with clean collodion films prepared under identical conditions and with blank areas adjacent to the lamellae on the same specimen film were always carried out.

OBSERVATIONS

The myelin lamellae are arranged concentrically in the sheath, and the transparent unit lamellae have an average thickness of 80 Å (7). In preparations obtained by dissociation or freezing sectioning of the myelin sheath



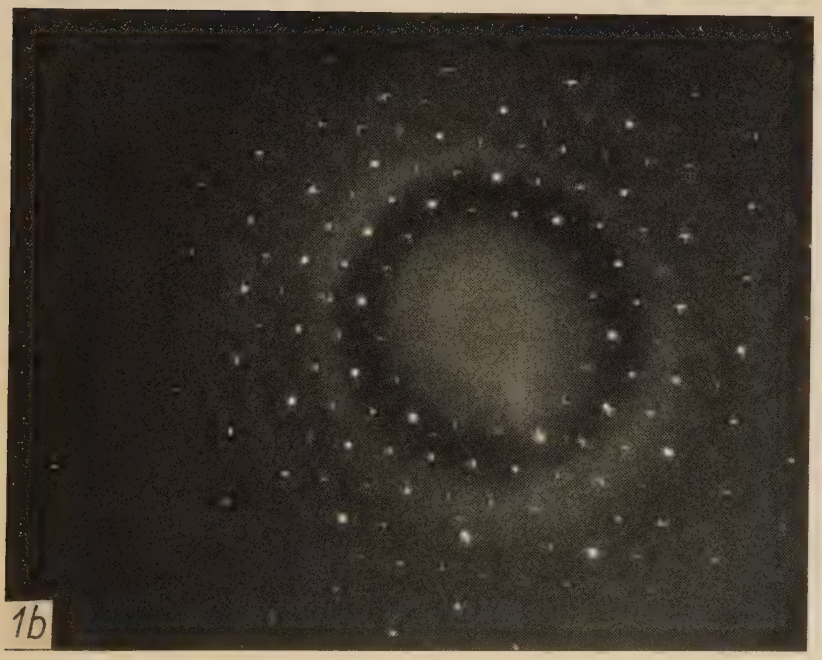
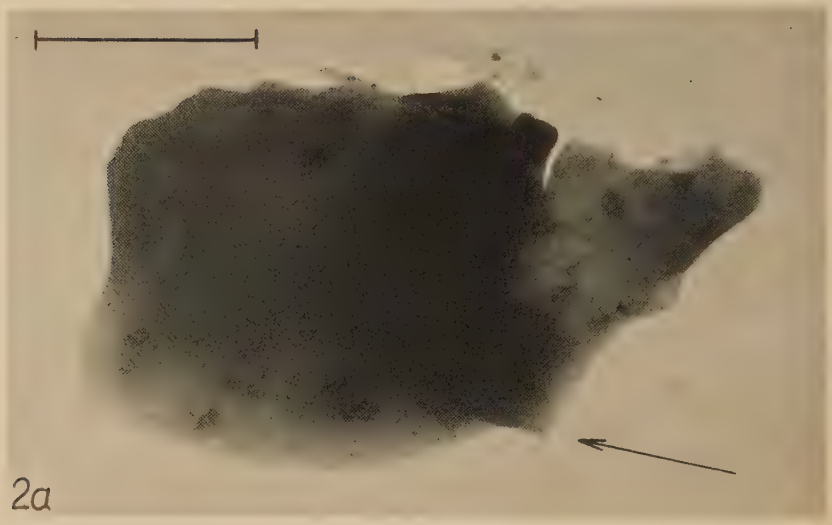


Fig. 1a. Thin slab of (4) osmium lamellae isolated from a dissociated rat sciatic nerve fibre fixed with 1 per cent buffered (pH 7.2) osmic acid solution for 7 hours, on collodion supporting film. These osmium lamellae produced the diffraction pattern shown in Fig. 1b. Magnification: 12 000 ×.

Fig. 1b. Electron diffraction cross-grating pattern obtained from the lamellae of Fig. 1a, using the specimen in the object position of an RCA EMU microscope fitted with a condenser aperture of 200 microns and an intermediate lens.



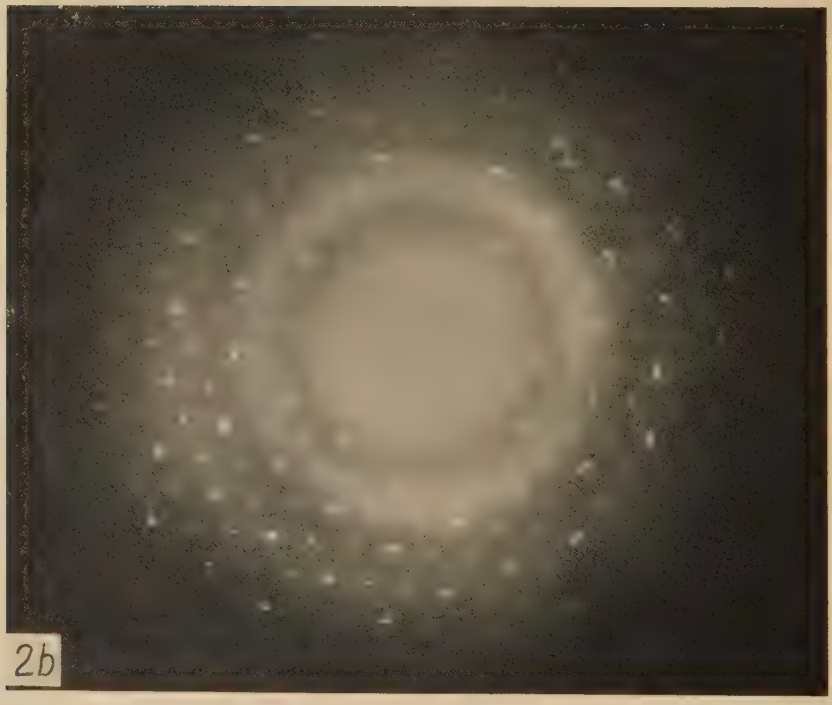


Fig. 2a. Thick pile of osmium lamellae isolated from a dissociated rat sciatic nerve fibre fixed with 1 per cent buffered (pH 7.2) osmic acid solution for 7 hours, on collodion supporting film. (The arrow indicates the edges of at least 7 lamellae). This pile of osmium lamellae is responsible for the diffraction pattern shown in Fig. 2b. Magnification: 30 000 ×.

Fig. 2b. Electron diffraction cross-grating pattern with multiple spots obtained from the lamellae pile of Fig. 2a, using the specimen in the object position of an RCA EMU electron microscope with a condenser aperture of 200 microns and an intermediate lens.

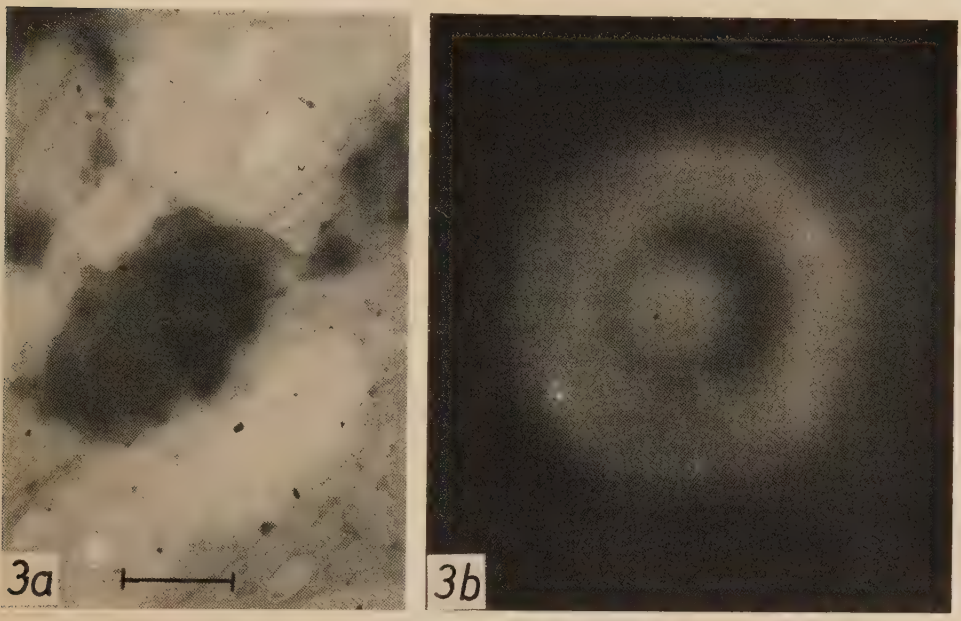


Fig. 3a. Pile of lamellae isolated from a dissociated rat sciatic nerve fibre fixed with 10 per cent buffered formalin solution. Magnification: 15 000 ×.

Fig. 3b. Electron diffraction pattern obtained from the field shown in Fig 3a, which is difficult to reproduce here because of the weak diffraction spots. Specimen in the object position of the electron microscope with a 200 microns condenser aperture and intermediate lens.

they generally occur stacked up in piles of 3–10 lamellae with stratified edges and a granular surface structure. Although the osmium lamellae closely resemble the myelin lamellae, they are generally thinner, have a smooth surface, angular edges and exhibit the described diffraction phenomena. Thin slabs of not more than 3–6 undistorted, clean osmium lamellae from rat sciatic nerve fibres fixed with osmium and lying flat on the collodion specimen film (Fig. 1 a) give strong, typical cross-grating patterns (Fig. 1 b), consisting of sharp single spots of variable size and intensity which are arranged symmetrically around the undeflected spot. This brilliant pattern of spots stands out against the background of diffuse rings produced by the collodion supporting film. Thicker piles of osmium lamellae (Fig. 2 a) produce similar cross-grating patterns with rows of double or multiple spots (Fig. 2 b) which gradually broaden as the piles become thicker, until they mark the stark outlines of a hexagonal pattern. Spot intensifications resembling Laue zone formations are occasionally observed in the thicker piles which are barely transparent to the electron beam. Kikuchi

lines have been found in a few cases only, mainly because of difficulties experienced in examining thicker piles, which do not stand up long under the intense electron beam. Lamellar structures from osmium fixed nerve fibres of the frog give essentially the same type of cross-grating diffraction patterns. If the intensity of the electron beam is increased, a peculiar scintillation of the cross-grating pattern is observed which results from brief intensification or extinction of the single spots. This phenomenon runs parallel with variations in the extinction contour pattern, and with changes in the position and configuration of the reflection images which show up in the electron microscope image of the osmium lamellae. These reflection images consist of diffuse light patches or lines lying on both sides of the lamellae, which are displaced laterally as the image is defocused. The intense electron bombardment rapidly volatilizes or distorts the osmium lamellae, leading finally to a diffuseness or obliteration of the image and to a concomitant weakening and extinction of the diffraction pattern which is clearly due to contamination of the irradiated area with a (carbonaceous) deposit (6). Only piles of osmium lamellae produce the described cross-grating patterns, since myelin lamellae which can be recognized by their typical granular surface structure do not give diffraction patterns. Neither do pieces of the compact granular neurilemma membrane, bundles of collagen fibrils or other components of the nerve fibre, all of which are osmium fixed, give rise to any detectable electron diffraction patterns. When two osmium lamellae piles of different thickness are close together, the transition from the single spot to the multiple spot type of cross-grating pattern is clearly discernible as the specimen is shifted. Displacement of the beam to an adjacent area of the collodion film immediately abolishes the pattern, and even prolonged irradiation of the collodion film will not change its characteristic diffuse ring pattern. Crystalline lamellae which are covered with osmium precipitates or other dense granules give easily recognizable combined cross-grating and ring patterns formed of numerous spots. Formalin fixed lamellar structures (Fig. 3 a) from rat or frog sciatic nerve fibres give irregular diffraction patterns (Fig. 3 b), which are however much weaker than the corresponding patterns from osmium fixed lamellae. Only a few sets of diffraction spots are visible in these patterns, which are rapidly extinguished as the lamellae suffer distortions or volatilize under the electron beam. This greater sensitivity of the formalin fixed lamellae and the lack of well preserved specimens may account for the comparatively few diffraction patterns recorded in these preparations. Moreover, it is possible that crystalline precipitates associated with the lamellar structures in formalin fixed preparations may have contributed to the diffraction patterns recorded.

DISCUSSION

At first the real lamellar structures of the myelin sheath were often confused with these crystalline osmium lamellae due to their striking resemblance, specially in dissociated preparations of the myelin sheath where both occur close together in large numbers. Subsequently however, crystalline lamellar structures of the same type as the osmium lamellae have been found in nerve lipid extracts treated with osmium, and among the precipitates produced during osmium fixation of nerve. It seems probable therefore that the osmium lamellae represent osmium inclusions formed in the nerve fibres during the process of fixation with osmium tetroxide solutions.

Without the arrangement described here for recording electron diffraction patterns of restricted specimen areas in the electron microscope, the crystalline osmium lamellae would be difficult to identify. The characteristic extinction contours and reflection images of the crystalline lamellae are not always clearly visible, and in unshadowed preparations the distinguishing granular surface structure of the myelin lamellae is not apparent. An interpretation of the patterns is not possible at this preliminary stage of the investigation, but experiments are being carried out to determine whether the crystalline lamellae represent osmium compounds of specific (lipid) components of the myelin sheath, or ordinary osmium fixation products.

REFERENCES

1. Ardenne, M. von, *Z. Physik*, **117**, 515 (1941).
2. Boadway, J. D., *J. Applied Phys.*, **22**, 104 (1951).
3. Boersch, H., *Z. Physik*, **116**, 469 (1940).
4. —— *ibid.*, **118**, 706 (1942).
5. —— *ibid.*, **121**, 746 (1943).
6. Davidson, N., and Hillier, J., *J. Applied Phys.*, **18**, 499 (1947).
7. Fernández-Morán, H., *Exptl. Cell Res.*, **1**, 309 (1950).
8. Finch, G. I., Quarrell, A. G., and Wilman, H., *Trans. Faraday Soc.*, **31**, 1051 (1935).
9. Heidenreich, R. D., *J. Applied Phys.*, **20**, 993 (1949).
10. Heidenreich, R. D., and Sturkey, L., *ibid.*, **16**, 97 (1945).
11. Hillier, J., *ibid.*, **21**, 785 (1950).

BRIEF NOTES

EINE APPARATUR ZUR MESSUNG DES ULTRAVIOLETT-DICHROISMUS VON ZELLSTRUKTUREN

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Gerichtete Nukleinsäure- und Eiweissmoleküle zeigen im ultravioletten Licht Dichroismus. Caspersson (1) hat erstmals diese Eigenschaft benützt, um den Orientierungsgrad solcher Moleküle in Zellstrukturen zu bestimmen. Meistens sind hier aber die Effekte derart klein, dass ihre Messung mit den gebräuchlichen mikrospektroskopischen Methoden Schwierigkeiten bereitet. Es wurde deshalb eine spezielle Apparatur mit der erforderlichen Empfindlichkeit für Dichroismus-Untersuchungen entwickelt.

Die Messung der Absorption mit polarisiertem Licht in zwei aufeinander senkrecht stehenden Schwingungsrichtungen erfolgt auf photoelektrischem Wege. Hierbei wird die zu messende Objektstelle mit dem Ultraviolett-Mikroskop auf eine in geeigneter Entfernung aufgestellte Photozelle projiziert. Zur Messung der Lichtschwächung kann das Objekt durch eine besondere Verschiebungsanordnung aus dem Strahlengang entfernt und wieder in die ursprüngliche Lage zurückgebracht werden (2). Neben einer hohen Messempfindlichkeit ist für die bezweckten Untersuchungen wichtig, dass die Messung der zwei Absorptionen in rascher Folge bei kurzer Bestrahlungsdauer geschieht, da sich die Präparate bei der intensiven Ultraviolett-Bestrahlung rasch verändern können.

Folgende Anordnung hat sich als geeignet erwiesen: Das Polarisationsprisma (Analysator) rotiert mit fünf bis zehn Umdrehungen pro Sekunde. Die Beleuchtungsstrahlen werden vollständig depolarisiert, so dass ohne Objekt ein konstanter Lichtstrom auf die Photozelle fällt. Befindet sich ein dichroitisches Objekt im Strahlengang, so entsteht ein sinusförmiger Lichtstrom (mit zwei Perioden pro Umdrehung des Analysators), die Photozelle erzeugt einen entsprechenden Wechselstrom. Dieser kann in einem Oszillographen beobachtet werden.

Für quantitative Untersuchungen wird eine Anordnung, bestehend aus Wechselstromverstärker, Gleichrichter und Galvanometer verwendet. Als Gleichrichter dient ein mit dem rotierenden Analysator gekoppelter Umschalter (Scheibe mit Schleifkontakten). Der Verstärker zerlegt den eintretenden Wechselstrom in zwei Wellen, welche gegeneinander eine Phasenverschiebung von einer halben Wellenlänge besitzen (3). Ist der Umschalter mit dem Analysator so synchronisiert, dass er im Nullpunkt jeder Schwingung abwechslungsweise von der einen Welle auf die andere umschaltet, so entsteht ein zerhackter Gleichstrom (Abb. 1). Dieser wird ausgeglättet und kann mit einem Galvanometer gemessen werden. Erfolgt die Um-

schaltung nicht im Nullpunkt der Schwingung, so entsteht ein schwächerer oder kein Messtrom. Dieser Umstand gestattet in einfacher Weise die Richtungen der zwei Absorptionen im Objekt zu bestimmen. Man hat dazu nur die Schleifkontakte des Umschalters zu drehen bis zum maximalen Galvanometerausschlag. Aus der einmal geeichten Stellung der Schleifkontakte ergibt sich dann die Richtung der grösseren bzw. kleineren Absorption. Die beschriebene Gleichrichteranordnung besitzt noch einen weiteren, für die Messgenauigkeit entscheidenden Vorteil. Sie gestattet nämlich, störende Polarisationserscheinungen in der Optik- und Photozelle, so wie Zentrierfehler des Analysators weitgehend unschädlich zu machen, da alle Ströme von anderer Frequenz oder Phase als der zu messende Strom nicht oder nur in unbedeutendem Masse gleichgerichtet werden. Absorptionsunterschiede bis zu etwa 0,5 Prozent sind noch nachweisbar.

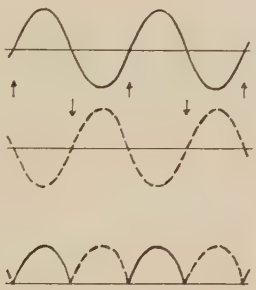

Abb. 1. Prinzip der Gleichrichtung des Messtromes.

Mit Hilfe einer Kompensationsmethode kann zunächst bei feststehendem Objekt das Verhältnis der zwei Absorptionen genau bestimmt werden. Hierzu dient ein dreh- und neigbarer Quarzplattensatz, in welchem eine der beiden polarisierten Wellen durch Reflexion messbar geschwächt werden kann. Die noch erforderliche Messung einer der beiden Absorptionen geschieht durch Einschalten eines zweiten Polarisationsprismas, so dass auch ohne Präparat ein Wechselstrom entsteht. Die Absorption ergibt sich dann aus dem Verhältnis des Galvanometerausschlages bei ein- bezw. ausgeschaltetem Objekt (bei Objekten mit starkem Dichroismus werden beide Absorptionen in dieser Weise bestimmt, da dann der für kleine Effekte bestimmte Kompensator nicht mehr ausreicht). Hierbei wirken sich natürlich Ungenauigkeiten in der Objektverschiebung, Schwankungen der Lichtquelle etc. auf die Messgenauigkeit aus (zur Korrektion von Intensitätsschwankungen der Lichtquelle dient ein besonderer Strahlengang mit einer zweiten Photozelle und zugehörigem Galvanometer). Da aber das Verhältnis der gesuchten zwei Absorptionen genau bekannt ist, ist auch ein schwacher Dichroismus mit ausreichender Genauigkeit nachweisbar. Der ganze Messvorgang dauert bei jeder Wellenlänge nur wenige Sekunden. Die Verwendung von Spiegeloptik im Mikroskop ermöglicht das Einstellen im sichtbaren Licht, so dass das Präparat nicht unnötig lange der starken Strahlung ausgesetzt werden muss.

Der Aufbau der Apparatur geht aus Abb. 2 hervor.

Von den bisher durchgeführten Untersuchungen sollen hier einige noch ganz kurz

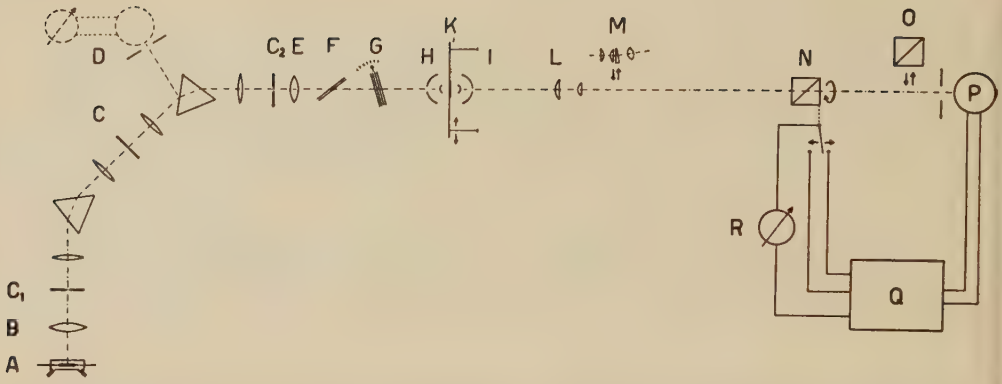

Abb. 2. Schema der Apparatur für Dichroismus-Messungen. A Quecksilberdampf-Superhochdrucklampe (Philips SP 500). B Quarzlinse. C Doppelmonochromator mit Eintrittsspalt C_1 und Austrittsspalt C_2. D Photozelle mit Galvanometer zur Überwachung von Lampenschwankungen. E Quarzlinse. F Quarzplatten zur Depolarisierung des Lichtes. G Quarzplattensatz zur Messung des Absorptionsverhältnisses (Kompensator). H, I Spiegelkondensor und Spiegelobjektiv, num. Apertur 0.72 (Bausch and Lomb). K Präparat mit Verschiebungsanordnung. L Okular aus geschmolzenem Quarz. M Fluoreszenzsucher. N Rotierendes Polarisationsprisma, mit dem elektrischen Umschalter verbunden. O Einschwenkbares Polarisationsprisma für die Absorptionsmessung. P Photozelle (Photomultiplikator, RCA 1P 28) mit Irisblende. Q Wechselstromverstärker. R Galvanometer.

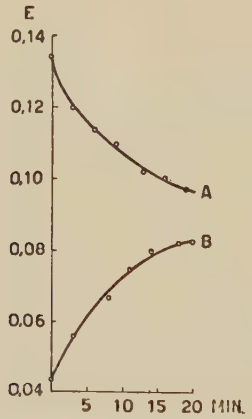

Abb. 3. Einfluss der Ultraviolett-Bestrahlung auf den Dichroismus von Thymonukleinsäurefäden. E Extinktion. Schwingungsrichtung des polarisierten Lichtes bei A senkrecht, bei " B parallel zum Faden

erwähnt werden. Zuerst wurde der Dichroismus künstlich orientierter Thymonukleinsäure gemessen (Na-Thymonukleinat, Präparation Hammarsten). Ein mit dem Finger zu mikroskopisch dünnen Fäden ausgestrichenes und eingetrocknetes Gel zeigt einen starken Dichroismus mit der grösseren Absorption senkrecht zur Strichrichtung.

Der gefundene Maximalwert des Dichroismus, im Verhältnis der beiden Extinktionen ausgedrückt, beträgt bei 265 mμ 3,5. (Da die Messung nicht im parallelen Licht, sondern entsprechend der verwendeten Spiegeloptik mit einer num. Apertur von 0,72 im konvergenten Licht erfolgte, liegt der tatsächliche Wert noch etwas höher.)[1] Aus elektronenmikroskopischen Untersuchungen der Nukleinsäurefäden geht hervor, dass die Nukleinsäuremoleküle annähernd parallel angeordnet sein müssen. Weiter wurden Versuche gemacht, um die Einwirkung der Ultraviolett-Bestrahlung auf die Modellsubstanz zu prüfen. Es zeigte sich dabei, dass schon nach relativ kurzer Bestrahlungszeit eine Desorientierung der Nukleinsäuremoleküle eintritt. Ein Beispiel der Abnahme des Dichroismus mit zunehmender Bestrahlungszeit ist in Abb. 3 dargestellt. Diese Beobachtungen erklären möglicherweise früher gefundene (1), niedrigere Dichroismuswerte.

Eine Reihe von Messungen ist an Chromosomen durchgeführt worden. So konnte in den Chromomerenscheiben der Speicheldrüsenchromosomen von Chironomus ein schwacher Dichroismus der Nukleinsäurekomponente gemessen werden, dessen Vorzeichen und Grösse von der Art der Präparation abhängig ist. Über diese Untersuchungen soll an anderer Stelle ausführlich berichtet werden.

LITERATUR

1. Caspersson, T., *Chromosoma*, **1**, 605 (1940).
2 —— *J. Roy Microscop. Soc.*, **60**, 8 (1940).
3. Savitzky, A., and Halford, R. S., *Rev. Sci. Instruments*, **21**, 203 (1950).

[1] Später ausgeführte Messungen mit Quarz-Monochromaten und kleiner Beleuchtungsapertur ergaben einen Dichroismus von 4,6.

RECOVERY OF THE GOLGI APPARATUS FROM HOMOGENATES OF NORMAL MAMMALIAN LIVER

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During the past decade, several studies have been made of centrifuged liver homogenates, particularly with reference to their biochemistry (5, 7, 8, 11, 14). Centrifugation separates the homogenized components into nuclear, mitochondrial, the so-called microsomatic, and the residual cytoplasmic fractions. Although similar investigations have been carried out on other tissues, mammalian liver has received the bulk of the attention. In all of these studies, the mitochondria in centrifuged homogenates lodge in a fairly well defined layer, but the Golgi apparatus (Golgi bodies; neutral red staining material; lipo-protein bodies; lipochondria), which in either fixed or living preparations nearly equals and sometimes apparently exceeds the total mitochondrial content in volume, is not identified. In view of the known or suspected importance of the Golgi or neutral red staining material in cellular processes (1, 4, 6, 10, 12) and its abundance in almost all animal cells, it is noteworthy and unfortunate that this cellular component has been overlooked so consistently in homogenized tissue.

Recently, Dr. Seifter furnished us with fresh normal liver homogenates from Wistar white rats and a search was made for the Golgi material in the four fractions. Tests were made with Sudan black B, osmium tetroxide and Nile blue sulphate in identifying the Golgi lipids. Lipids were detected in all four fractions, as one would expect, for as is well known, these fractions are by no means pure. However, the bulk of the lipids, estimated at between 75 and 90 per cent, was lodged in the residual cytoplasmic fraction. This too is, of course, to be expected since this is the lightest layer of the homogenate.

Positive identification of the lipid spheres as either the Golgi apparatus, or as its lipid component, was confirmed by observing the peculiar effect of Nile blue sulphate upon them. When a drop of Nile blue (1 per cent in distilled water) was added to 2 ml of homogenate, the dye not only colored the lipid spheres, but also caused their aggregation into clumps of fairly uniform size which subsequently became distorted, while under observation, so that typical Golgi figures of the classical network type were produced (Figs. 1, 2). Curiously, each individual Golgi network produced in this fashion in this isolated material was of a size approximately equal to that encountered in individual cells of fixed preparations. Gross aggregation of lipid granules was not observed and each individual network appeared to be formed of not more than 25 to 30 lipid spheres. No cytologist, familiar with the typical Golgi network configuration of the fixed liver cell, would hesitate to identify these figures as "Golgi

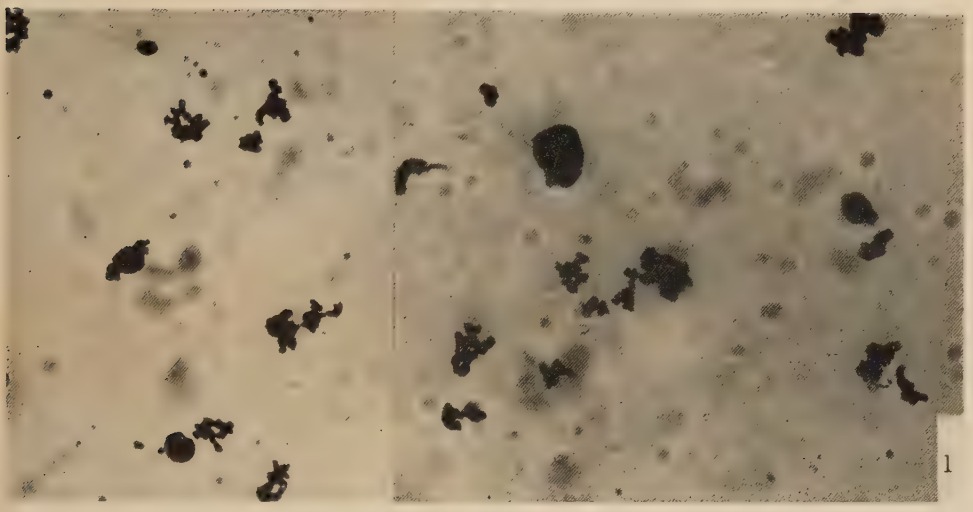

Fig. 1. Golgi networks artificially produced extracellularly by treating the residual cytoplasmic fraction of centrifuged normal rat liver homogenate with Nile blue sulphate. Photo. 700 ×.

apparatus" were they encountered in a permanent fixed preparation prepared according to the standard methods for the demonstration of this inclusion.

There are several points of value in these observations:

1. The Golgi networks illustrated in Figs. 1 and 2 are believed to be the first ever to have been observed in any quantity isolated from the cell.

2. These networks have been artificially produced by treating isolated lipid spheres from liver with Nile blue sulphate. This is strong presumptive evidence that the Golgi network configuration of the fixed liver cell is also artificially produced and is not a natural feature of the cell, as maintained recently by Bensley (3), and by many others in the past.

3. Since, according to Muntwyler, Seifter and Harkness (11), the residual cytoplasmic fraction contains 47 per cent of the phosphorus in the liver homogenate, these lipids are undoubtedly largely phospholipids, a view strongly supported by the detailed studies of Baker (1), and more recently those of Palade and Claude (13), with regard to the chemical nature of the Golgi apparatus lipids.

4. However, Muntwyler, Seifter and Harkness (11) find that 36 per cent of the total PNA and 55 per cent of the total nitrogen of the homogenate also occurs in this fraction, suggesting the possibility that these substances may also be a part of, or contribute to, the total Golgi complement of the normal liver cell. This possibility has been indicated on other grounds for other tissues by Baker (2), Hirsch (6), Lavin and Pollister (9), Oliver (12), and others (15), in opposition to the view of Palade and Claude (13) who regard the Golgi material simply as phospholipid without other chemical affiliations.

5. As is evident in the illustrations, no myelin figures were observed in the homogenate, suggesting that although the Golgi lipids may produce myelin figures when

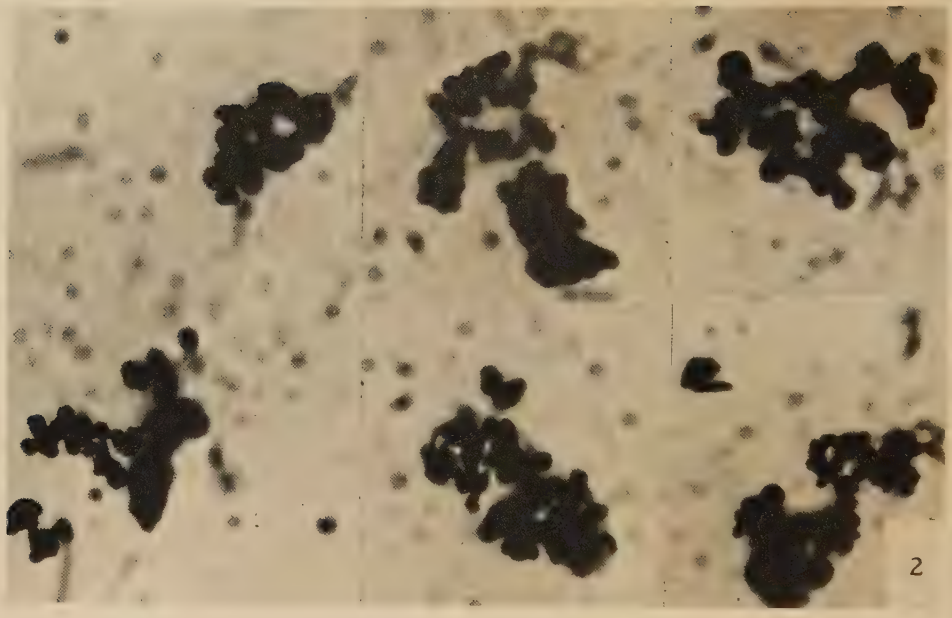

Fig. 2. Same, greatly enlarged to show details of individual networks. Note absence of myelin forms and "chromophobic" elements of the Golgi complex. Photo. 3 200 ×.

treated with ethyl alcohol and other appropriate reagents, as was done by Palade and Claude (13), the general statement that the Golgi apparatus is a myelin figure is open to modification. The observations of Palade and Claude are, however, of considerable value in that they appear to offer an explanation for those cases where the Golgi apparatus in some tissues has been described as a "canalicular" structure. On the other hand, the interpretation of the Golgi apparatus as a system of canals has not found much favor, at least for the majority of tissues, in recent years. It is also conceivable that the phospholipid alone will not produce the myelin forms.

6. No "chromophobic" component or "internum" of the Golgi complex (6, 16) has been identified in these preparations. Since this component is presumed to be primarily protein, it seems probable that in centrifuging some or all of this component of the complex has been separated from the lipid portion and has become lodged in one of the heavier layers of the centrifugate.

REFERENCES

1. Baker, J. R., *Quart. J. Micr. Sci.*, **85**, 1 (1944).
2. —— *Proc. Linnean Soc. London*, **162**, 67 (1950).
3. Bensley, R. R., *Exptl. Cell Res.*, **2**, 1 (1951).
4. Bourne, G., Cytology and Cell Physiology. Clarenden, Oxford, 1951.
5. Claude, A., *J. Exptl. Med.*, **80**, 19 (1944).

6. Hirsch, G. C., Form- und Stoffwechsel der Golgi-körper. Borntraeger, Berlin, 1939.
7. Hogeboom, G. H., Claude, A., and Hotchkiss, R. D., *J. Biol. Chem.*, **165**, 615 (1946).
8. Hogeboom, G. H., Claude, A., and Palade, G. E., *ibid.*, **172**, 619 (1948).
9. Lavin, G. I., and Pollister, A. W., *Biol. Bull.*, **83**, 299 (1942).
10. Leuchtenberger, C., and Schrader, F., *Proc. Natl. Acad. Sci.*, **36**, 677 (1950).
11. Muntwyler, E., Seifter, S., and Harkness, D. M., *J. Biol. Chem.*, **184**, 181 (1950).
12. Oliver, J., *J. Mt. Sinai Hospital*, **15**, 175 (1948).
13. Palade, G. E., and Claude, A., *J. Morphol.*, **85**, 71 (1949).
14. Schneider, W. C., *J. Biol. Chem.*, **176**, 259 (1948).
15. Worley, L. G., *Ann. N. Y. Acad. Sci.*, **47**, 1 (1946).
16. Xeros, N., *Nature*, **167**, 448 (1951).

THEORETICAL CONSIDERATIONS OF THE RATE OF DEHYDRATION BY HISTOLOGICAL FREEZING-DRYING[1]

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INTRODUCTION

Most freezing-drying apparatus described in the literature require a relatively long time (up to 1 week) for the complete dehydration of the tissue. While the results may be very satisfactory from both a morphological and histochemical standpoint, the long periods involve an inconvenience in laboratories where the freezing-drying technique has become a routine method of tissue treatment. In designing a freezing-drying apparatus it therefore becomes important to know which variables will influence the rate of drying. It is the purpose of this paper to discuss the most important of these factors and to give an indication of the relative quantitative contribution of each. Packer and Scott (4) have previously given equations for the rate of dehydration, but the following treatment is somewhat more complete. To simplify the analysis, the process of drying has been divided into two steps which will be considered separately: A. The evaporation of water from the frozen sample. B. The removal of the evaporated water.

THEORETICAL ANALYSIS

A. *The rate of Evaporation*

According to the kinetic theory of gases (3): $Q = p\sqrt{\frac{M}{2\pi RT}}$ where Q = mass (g) of gas striking 1 cm^2 per sec, p = pressure (dynes/cm^2). M = molecular weight, and T = absolute temperature. From this equation an expression for the rate of evaporation can be derived. When p is equal to the saturated vapor pressure (p_T) for a given temperature (T), equilibrium will be attained, i.e. the rate of evaporation will equal the rate of condensation. Thus, the rate of evaporation (Q_e) is given by the following equation: $Q_e = P_T\sqrt{\frac{M}{2\pi RT}}$. Q_e is only dependent on the kinetic energy of the water molecules in the sample, or, in other words, the temperature. Thus, the above equation gives the rate of evaporation even if equilibrium is not attained. To get the net rate of evaporation, the rate of condensation (Q_c) must be subtracted from the value

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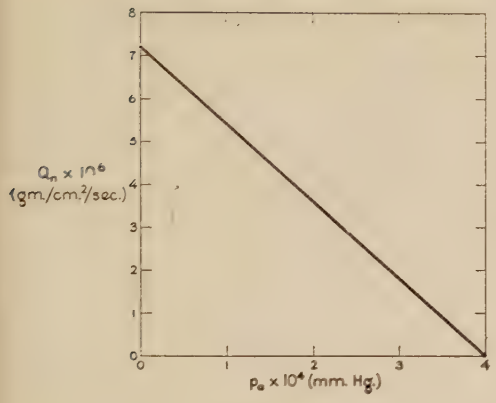

Fig. 1.

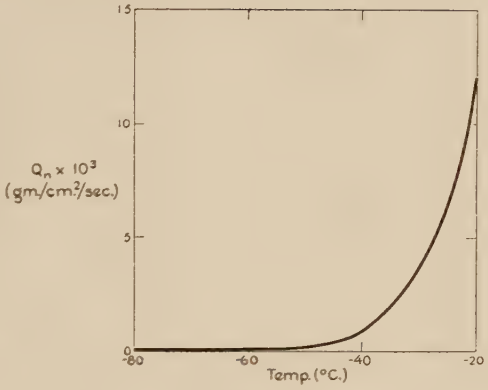

Fig. 2.

Fig. 1. Rate of evaporation of water at —80° C as a function of pressure.
Fig. 2. Rate of evaporation of water as a function of temperature at pressures below 10^{-5} mm Hg.

of Q_e: $Q_c = p_a \sqrt{\frac{M}{2\pi RT}}$ where p_a = the actual pressure. The net rate of evaporation (Q_n) is then: $Q_n = (p_T - p_a)\sqrt{\frac{M}{2\pi RT}}$. If the proper values of the constants are introduced, the pressures are expressed in mm Hg, and the molecular weight is set equal to 18 (H_2O), this equation becomes: $Q_n = 0.25\,(p_T - p_a)\frac{1}{\sqrt{T}}$. It can thus be seen that the rate of evaporation of water from the sample depends on two factors, namely p_a and T (p_T is a function of T and is not an independent variable).

1. *Effect of pressure.*

Assume that the sample is kept at the temperature of dry ice, while the pressure is varied, e.g. by using pumps of different speeds. The conditions are: $T = 193°$ K, $p_T = 0.00040$ mm Hg, which gives: $Q_n = 1.8 \times 10^{-2}\,(4.0 \times 10^{-4} - p_a)$. The plot of the last equation is given in Fig. 1. It can be seen that the lower the value of p_a, the greater the rate of evaporation. There is, however, little to be gained by using pressures below 10^{-5} mm Hg.

2. *Effect of temperature.*

If p_a is assumed to be so small that it can be neglected compared to p_T, Q_n is given by the following formula: $Q_n = 0.25\, p_T \frac{1}{\sqrt{T}}$. Fig. 2 gives the graphic representation of this equation. While the graph shows that the rate of evaporation increases rapidly with temperatures, it must be remembered that this means of improving the efficiency is limited by the fact that the sample must be kept in the frozen state throughout the dehydration. However, it is important to note the large absolute increase in Q_n obtained by employing temperatures above —40° C.

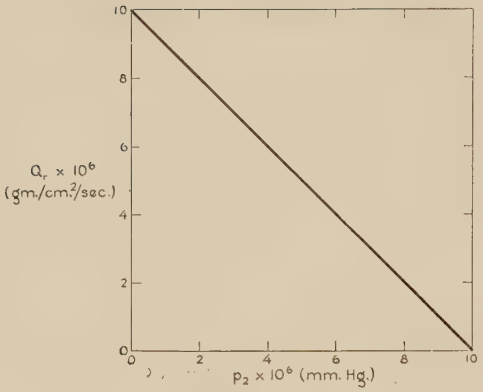

Fig. 3.

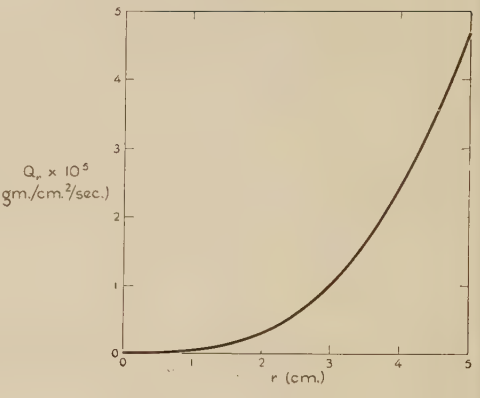

Fig. 4.

Fig. 3. Rate of removal of evaporated water as a function of partial pressure at trap.
Fig. 4. Rate of removal of evaporated water as a function of radius of tubing of freezing-drying unit.

B. *The Rate of Removal of Evaporated Water*

The rate of removal of the evaporated water (Q_r) can be calculated from Knudsen's equation (2): $Q_r = \frac{4}{3}\sqrt{\frac{2\pi M}{RT}} \times \frac{r^3}{L}(p_1 - p_2)$, where r = radius of the tubing through which the gas flows, L = length of path from sample to trap, $p_1 = p_a$, and p_2 = partial pressure of water at the trap. This equation holds only if r is small compared to the mean free path. In the following discussion it will be assumed that p_1 is 10^{-5} mm Hg. At this pressure the mean free path of water molecules is 600 cm. As the greatest value of r considered is 5 cm, the use of the above equation is justified.

If p_1 and p_2 are expressed in mm Hg, M equals 18 (H_2O), and T is 193° K (dry ice), Knudsen's equation becomes: $Q_r = 0.15\frac{r^3}{L}(p_1 - p_2)$.

1. *Effect of partial pressure of water at the trap.*

If it is assumed that the pumps bring p_1 down to 10^{-5} mm Hg, that r is 3 cm, and L is 4 cm, one obtains: $Q_r = 1.0\ (10^{-5} - p_2)$. Fig. 3 gives the plot of this equation. From this it is clear that it is advantageous to use a liquid nitrogen trap instead of phosphorus pentoxide, as the partial pressure of water over phosphorus pentoxide at room temperature is 10^{-5} mm Hg, while in a liquid nitrogen trap it is about 10^{-24} mm Hg (1).

2. *Effect of radius of tube holding sample.*

If p_2 is assumed to be so small that it can be neglected compared to p_1 (1×10^{-5} mm Hg) and if L still is 4 cm, an equation relating Q_r and r is obtained: $Q_r = 3.75 \times 10^{-7} r^3$. Fig. 4 gives the graph corresponding to this equation. This shows the radius to be a great factor. However, it should be kept in mind that the water evaporates

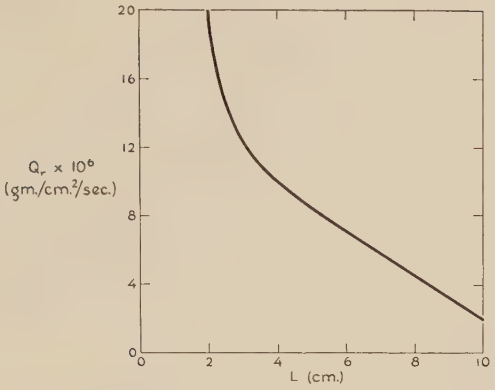

Fig. 5. Rate of removal of evaporated water as a function of distance between sample and trap.

not over the entire cross section of the tube but only from the limited area of the tissue surface. But this equation also determines the pumping speed, which, as has been shown earlier, must be high to lower the value of p_a. It is, therefore, undoubtedly advantageous to have a radius as great as 3 cm.

3. *Effect of length of path from sample to trap.*

If all conditions are maintained as previously described except that r is fixed at 3 cm and L is varied, the following equation is obtained: $Q_r = \frac{4.0}{L} \times 10^{-5}$. Fig. 5 is a graphic representation of this equation. Many designs of freezing-drying apparatus now in use employ paths considerably greater than 10 cm, while it can be seen that there is a definite advantage in reducing it as much as possible. About 4 cm is probably a practical limit.

DISCUSSION

In the preceding analysis the following factors have been varied independently: the temperature of the sample, the pressure over the sample, the partial pressure of water at the trap, the radius of the tubing of the freezing-drying unit, and the length of the path from the sample to the trap. It must, however, be borne in mind that all these factors are interdependent. It is, for example, futile to try to decrease the drying time by increasing the temperature of the sample, if the apparatus is so designed that the rate of removal of the evaporated water will not be optimal. Otherwise, the pressure over the sample will increase and the net rate of evaporation will actually decrease despite the higher temperature. It must also be realized that the above calculations, while giving the optimum values of certain variables, are useless in calculating the absolute time of dehydration, as many complex factors that apply to a practical case have not been and actually cannot be considered, e.g. the rate of diffusion of water vapor through the tissue and the cooling effect of evaporation.

SUMMARY

The dependence of the rate of dehydration by freezing-drying on the temperature of the sample, the pressure over the sample, the partial pressure of water at the trap, the radius of the tubing of the freezing-drying unit, and the length of the path from the sample to the trap has been discussed. From the analysis it can be concluded that a freezing-drying apparatus will be very efficient if it has: 1) pumps capable of giving a pressure of 10^{-5} mm Hg; 2) a sample temperature above —40° C; 3) a water condenser at approximately the temperature of liquid nitrogen; 4) a radius of the tubing of the freezing-drying unit greater than 3 cm; and 5) a path between the sample and the condenser less than 4 cm.

ACKNOWLEDGEMENT

The author wishes to thank Dr. David Glick, who started his interest in the freezing-drying technique, for many helpful discussions.

REFERENCES

1. International Critical Tables, Vol. III, p. 385.
2. Kennard, E., Kinetic Theory of Gases. New York, p. 305, 1938.
3. MacDougall, F. H., Physical Chemistry. New York, p. 52, 1944.
4. Packer, D. M., and Scott, G. H., *J. Tech. Methods*, **22**, 85 (1942).

THE IMPORTANCE OF ADEQUATE STIMULATION FOR THE CHEMICAL COMPOSITION OF GANGLION CELLS

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It has been shown that adequate stimulation of a neuron causes an increased production of pentose nucleoproteins in the nerve cell (4, 5). The investigations were carried out with biophysical methods on adult nerve cells from the spinal ganglions, nuclei of the spinal cord, the cerebellum and the VIIIth nerve. The present investigation deals with the following problem. What is the importance of adequate stimulation, applied to a particular neuron at a certain stage, for its development into a structurally, chemically and functionally complete unit?

Eyes from rabbits were used as the experimental material. The large ganglion cells of the retina can be completely excluded from adequate stimulation and are therefore ideal objects. The rabbits were born in a dark box and lived in complete darkness for ten weeks. Other animals were subjected to adaptation to darkness for varying periods. The material was fixed in Carnoy's solution and embedded in paraffin. The quantitative determinations were made by means of microradiography according to Engström and Lindström (3). Certain modifications for quantitative purposes and for the determination of pentose nucleic acids were introduced at our department (1, 2). The lipids of the cells were extracted with alcohol and chloroform. The pentose nucleic acids were obtained as a difference in mass after digestion of the section with ribonuclease free from proteolytic activity. The results are given in Table I.

The figures for the control animals stimulated with ordinary daylight show that the total amount of dry substance in the lipid-extracted ganglion cells consisted of pentose nucleic acids and proteins in the proportion of around 1 : 1. The amount of nucleic acids per μ^3 in the cytoplasm does not seem to differ from that obtained from other motor and sensory ganglion cells of control animals.

Ganglion cells from dark-adapted eyes did not contain measurable amounts of pentose nucleic acids. Adaptation to darkness for 3 hours was sufficient to cause their disappearance. After digestion with ribonuclease, an amount of cell material remained which, with the exception of 5–7 per cent, can be regarded as consisting of protein substances. This mass was of the same order of magnitude as that remaining after digestion of stimulated cells with ribonuclease. It may be pointed out that adaptation to darkness for 3 months did not result in any measurable decrease in this cell component.

The retinal ganglion cells of animals whose eyes had not been exposed to adequate stimulation were also found to lack measurable amounts of pentose nucleic acids.

TABLE I

		Retinal ganglion cells	
		Cytoplasm	Nucleus
Control rabbit living in ordinary daylight	Weight in mg/μ^3 after extraction of lipids	$1.6 \cdot 10^{-9}$	$1.4 \cdot 10^{-9}$
	Weight in mg/μ^3 after digestion with ribonuclease	$0.8 \cdot 10^{-9}$	$0.7 \cdot 10^{-9}$
	Nucleic acids. Weight in mg/μ^3	$0.8 \cdot 10^{-9}$	$0.7 \cdot 10^{-9}$
Rabbit living in ordinary daylight. Adapted to darkness for 3 hours	Weight in mg/μ^3 after extraction of lipids	$0.8 \cdot 10^{-9}$	$0.6 \cdot 10^{-9}$
	Weight in mg/μ^3 after digestion with ribonuclease	$0.8 \cdot 10^{-9}$	$0.6 \cdot 10^{-9}$
	Nucleic acids. Weight in mg/μ^3	$0.0 \cdot 10^{-9}$	$0.0 \cdot 10^{-9}$
Rabbit living in ordinary daylight. Adapted to darkness for 3 months	Weight in mg/μ^3 after extraction of lipids	$1.1 \cdot 10^{-9}$	$0.8 \cdot 10^{-9}$
	Weight in mg/μ^3 after digestion with ribonuclease	$1.1 \cdot 10^{-9}$	$0.8 \cdot 10^{-9}$
	Nucleic acids. Weight in mg/μ^3	$0.0 \cdot 10^{-9}$	$0.0 \cdot 10^{-9}$
Rabbit born in darkness. Living in complete darkness for 10 weeks.	Weight in mg/μ^3 after extraction of lipids	$0.1 \cdot 10^{-9}$	$0.07 \cdot 10^{-9}$
	Weight in mg/μ^3 after digestion with ribonuclease	$0.1 \cdot 10^{-9}$	$0.06 \cdot 10^{-9}$
	Nucleic acids. Weight in mg/μ^3	$0.0 \cdot 10^{-9}$	$0.01 \cdot 10^{-9}$

In this respect they resembled the corresponding cells from animals adapted to darkness. Other quantitative differences were, however, found. Thus, the whole cell mass was exceedingly small compared with that of control animals which had received adequate stimulation and whose eyes had functioned normally. As may be inferred from the table, the cell mass of the control animals, after digestion with ribonuclease, was approximately ten times greater than that of the cells from the animals born in darkness. This difference was mainly reflected in a difference in the protein content of the cytoplasm. Non-stimulated adult cells also fail to present the characteristics of embryonic nerve cells.

Thus, the aforementioned results show that the stimulation of retinal ganglion cells causes an intracellular production of pentose nucleic acids. Temporary lack of stimulation of the neuron causes a decrease in the amount of nucleic acids, but the remaining cell fraction, *i.e.*, the proteins, remains quantitatively intact. If the adult cell is entirely deprived of the adequate stimulation to which it is intended to be exposed and is never permitted to function, its chemical composition will remain incomplete.

REFERENCES

1. Brattgård, S.-O., and Hallén, O., *Biochim. et Biophys. Acta* (in press).
2. Brattgård, S.-O., and Hydén, H., *Exptl. Cell Res.* (in press).
3. Engström, A., and Lindström, B., *Biochim. et Biophys. Acta*, **4**, 351 (1949).
4. Hydén, H., *Acta Physiol. Scand. Suppl.* **17** (1943).
5. Hydén, H., in Genetic Neurology, edited by P. Weiss, p. 177, 1950.

INCORPORATION *IN VIVO* OF LABELED NITROGEN AND PHOSPHORUS INTO THE RIBONUCLEIC ACID OF CHICK LIVER CYTOPLASM FRACTIONS

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The present experiments were performed in connection with a study of the incorporation of N^{15}-glycine into the proteins of chick liver cell fractions (6).

Newly hatched chicks (White Leghorn) received an injection into one wing vein of 26.8 mg N^{15}-glycine (5 mg N^{15}, 61 per cent excess), or 0.1–0.4 mcs of radioactive phosphate. In the N^{15}-experiments, 4–6 chicks were used, and in the P^{32}-experiments, 6–12 chicks. The chicks were killed by decapitation 20–60 minutes later. The livers were immediately removed, dissected and washed in ice-cold Chambers-solution (2). They were homogenized, strained, and fractionated in the cold in the same manner as described previously (6), except that the supernatant fluids obtained by washing the mitochondria and microsome fractions were discarded. Thus, if granules were broken down by the washing procedures, the fragments would not interfere with the subsequent fractions.

The cell fractions were precipitated with ice-cold, 10 per cent trichloroacetic acid (TCA), centrifuged, and thoroughly washed once in ice-cold 2.5 per cent TCA, containing 0.1 N ordinary glycine (N^{15}-experiments) or NaH_2PO_4 (P^{32}-experiments), and then twice in pure, ice-cold, 2.5 per cent TCA. The lipids were removed by alcohol and alcohol-ether extractions. Finally, the nucleic acid nucleotides were brought into solution by means of a 10 minute treatment with 3 per cent TCA at 88–90° C (cf. 15).

In the N^{15}-experiments, the extracted nucleotides were precipitated at pH 2 by addition of 0.2 volumes of saturated mercuric acetate. After treatment with H_2S and reprecipitation with mercuric acetate, the nitrogen of the mercury nucleotides was determined and analyzed for excess N^{15}. Further reprecipitations of the nucleotides did not change the distribution of the isotope between the cell fractions, and this very simple isolation procedure thus seemed to be adequate for the present purpose.

In the P^{32}-experiments, the extracted and Hg-precipitated nucleotides were treated with H_2S and run through columns containing the cation exchange resin Dowex 50 (4). Distilled water and 0.1 N acetic acid were used for elution. The nucleotides were well separated, and were eluted in the following sequence: uridylic acid, guanylic acid, cytidylic acid, and adenylic acid. As is well known, the purine nucleotides are sensitive to acid hydrolysis (9). Because of the strongly acid properties of Dowex 50,

TABLE I

Uptake of N^{15} from Labeled Glycine into the Nucleic Acids of Chick Liver Cell Fractions. Per cent excess N^{15}.

Times after the injections of isotope	20 minutes	45 minutes	60 minutes
Cell Fluid	0.025	0.05	0.09
Microsomes	0.015	0.04	0.07
Mitochondria	0.010	0.03	0.05
(DNA from nuclei)	(< 0.01)	—	(0.03)

especially adenylic acid was obtained from the eluate in much reduced yield, and was excluded from the analyses. The different hydrolysis rates of the purine and pyrimidine nucleotides (9) was utilized in the analyses in the following way: The pyrimidine nucleotide fractions were divided into two parts. One part was hydrolysed with 10 N H_2SO_4 at 150° C for 15 hours. The other part was hydrolysed with N H_2SO_4 at 100° C for 60 minutes, and was used as a control. The guanylic acid fractions were also divided into two parts. One part was hydrolysed with N H_2SO_4 at 100° C for 60 minutes. The other part was used as a control without hydrolysis. Phosphate was determined according to the method of Martin and Doty (12, cf. 5). After phosphate readings, the activities of the same samples were determined according to Lindberg (10). The control values were subtracted before calculation of specific activities.

RESULTS

The uptake of N^{15}-labeled glycine into the nucleic acids of the chick liver cell fractions is illustrated by Table I. The values are lower than the corresponding protein values, communicated previously by one of the authors (6). A similar relation was earlier observed in the rat by Hammarsten *et al.* (1). The ribonucleic acid of the cell fluid fraction had the highest isotope content. As in the protein experiments (6), the microsomes showed a higher isotope uptake than the mitochondria. During the course of the present study, Jeener (7, 8) published experiments, in which the incorporation of radioactive phosphate into the nucleic acids of cell fractions was studied in both embryonic and adult cells. The distribution of the N^{15} isotope in our chick material corresponds to the behavior of the adult cell type in Jeener's experiments (cf. 13).

The uptake of radioactive phosphate into the nucleic acids of the cell fractions (Table II) was in large similar to the N^{15} uptake (cf. 11). Of especial interest was the distribution of the phosphate activity between the investigated nucleic acid nucleotides. If nucleic acids were built up directly from a supply of mononucleotides, it seemed quite probable that these mononucleotides would have different rates of renewal. Such differences would reasonably be expected at least between the purine and pyrimidine nucleotides. In P^{32}-experiments of short duration, isolated nucleic acid nucleotides would then have different specific activities.

Table II

Specific Activities of P^{32}-labeled Ribonucleic Acid Nucleotides from Chick Liver Cytoplasm Fractions. Counts/μg P/minute.

	Cell Fluid	Microsomes	Mitochondria
Uridylic Acid	5.5	[1]	[1]
Guanylic Acid	6.1	1.7	1.1
Cytidylic Acid	5.6	1.7	1.2
Uridylic Acid	7.3	[1]	[1]
Guanylic Acid	—	2.5	1.5
Cytidylic Acid	7.8	2.3	1.9

Table III

Specific Activities of Guanylic and Cytidylic Acids in Experiments with Whole Homogenate and Isolated Microsome Fractions.

	Guanylic Acid	Cytidylic Acid
Whole Homogenate	3.6	3.5
Microsomes	1.9	1.8
»	2.2	1.9

Table IV

Specific Activities of Uridylic, Guanylic, and Cytidylic Acids from Isolated Microsomes.

Uridylic Acid	3.4
Guanylic Acid	3.3
Cytidylic Acid	3.2

In the present experiments, the main stress was laid on a comparison between guanylic acid (purine nucleotide) and cytidylic acid (pyrimidine nucleotide), which were obtained from the Dowex 50 columns in a very pure state. Uridylic acid, on the contrary, passed straight through the columns (4) like ordinary phosphate esters. If a nucleic acid preparation contained traces of highly labeled acid phosphates, the uridylic acid fraction consequently got an abnormally high specific activity. The specific activity of the control (hydrolysed in N H_2SO_4 for 60 minutes as described above) then became especially high. Particularly the mitochondria, but often also the microsome preparations showed this complication.

In this series of experiments, no constant difference in specific activity was found between the guanylic and cytidylic acids (Tables II–IV). Experiments were performed

[1] Traces of highly labeled acid phosphates appeared in the uridylic acid fraction (cf. text).

both with whole homogenates and with isolated cell fractions. Moreover, the specific activity of the uridylic acid of the cell fluid fraction was always consistent with that of the other investigated nucleotides. In a preliminary experiment with the anion exchange resin Dowex A 1[1], in which the nucleotides were eluted in another sequence (3), the uridylic and adenylic acids had approximately the same specific activity as the cytidylic acid.

If ribonucleic acid is built up directly from a supply of mononucleotides, the present experiments suggest that in our material such mononucleotides have very similar rates of renewal. Another interpretation is that the ribonucleic acid molecules are not built up from mononucleotide units, but that the phosphate groups are successively transferred to the growing polynucleotide chain as the new links are connected. This latter alternative is in very good agreement with recent experiments by Roll, Brown, Di Carlo, and Schultz (14), who found that adenylic acid is less utilized than free adenine for the synthesis of ribonucleic acid in the rat.

REFERENCES

1. Bergstrand, A., Eliasson, N. A., Hammarsten, E., Norberg, B., Reichard, P., and v. Ubisch, H., *Cold Spring Harbor Symposia Quant. Biol.*, **13**, 22 (1948).
2. Claude, A., *J. Exptl. Med.*, **84**, 51 (1946).
3. Cohn, W. E., *J. Am. Chem. Soc.*, **71**, 2275 (1949).
4. —— *Science*, **109**, 377 (1949).
5. Ernster, L., Zetterström, R., and Lindberg, O., *Acta Chem. Scand.*, **4**, 942 (1950).
6. Hultin, T., *Exptl. Cell Res.*, **1**, 376 (1950).
7. Jeener, R., *Nature*, **163**, 837 (1949).
8. Jeener, R., and Szafarz, D., *Arch. Biochem.*, **26**, 54 (1950).
9. Levene, P. A., and Jorpes, E., *J. Biol. Chem.*, **81**, 575 (1929).
10. Lindberg, O., *Arkiv Kemi, Mineral., Geol.*, **23** *A*, No. 2 (1946).
11. Marshak, A., and Calvet, F., *J. Cellular Comp. Physiol.*, **34**, 451 (1949).
12. Martin, J. B., and Doty, D. M., *Anal. Chem.*, **21**, 965 (1949).
13. Novikoff, A. B., and Potter, V. R., *J. Biol. Chem.*, **173**, 233 (1947).
14. Roll, P. M., Brown, G. B., Di Carlo, F. J., and Schultz, A. S., *J. Biol. Chem.*, **180**, 333 (1949).
15. Schneider, W. C., *J. Biol. Chem.*, **161**, 293 (1945).

[1] Kindly supplied by the Dow Chemical Company.

THE CAROTENOID PIGMENTS DURING EARLY DEVELOPMENT OF THE EGG OF THE SEA URCHIN *PARACENTROTUS LIVIDUS*

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The eggs of the sea urchin *Paracentrotus lividus* contain an orange-red pigment in the form of small granules. As it will be shown in a forthcoming paper, the granules consist of at least two proteins conjugated with carotenoids. An analysis of the carotenoids of the eggs is also in progress in this laboratory.

This investigation was undertaken in order to find out whether any physiological role could be ascribed to the pigment granules of the egg. Monroy and Runnström (3) indeed have presented evidence that the material of the pigment granules of the egg of *Arbacia punctulata* participates in the hardening of the fertilization membrane.

The results described in this paper indicate that the amount of pigment decreases during the early stages of development of the egg, thus suggesting that it may be involved in some morphogenetic process.

TECHNIQUE

Since the quantity of pigment varies in the eggs of different females, the eggs of one single female were used for each experiment. From each batch of eggs a sample was collected before fertilization. The eggs were then fertilized and allowed to develop under continuous stirring. The experiments were carried out at +20° C. From the homogeneous suspension, samples were taken at different intervals with a graduated pipette, transferred to a 10 ml glass stoppered cylinder and allowed to settle. The supernatant water was withdrawn, about 3 ml of ethanol and 7 ml of light petroleum ether (b.p. 30°–50°) were added and the mixture was shaken vigorously. In this way most of the pigment passed into the petroleum ether. After about one hour the petroleum ether extract was withdrawn and the residual pigment in the alcoholic phase was transferred quantitatively in ether by addition of water (2). The eggs were now completely colourless. The ether extract was dried over anhydrous Na_2SO_4 under a stream of N_2. The residue was taken up in a small amount of petroleum ether and mixed with the first extract. The volume of the extract was then concentrated to 4 ml under a stream of N_2 and the spectrum was read in the Beckman

[1] Centre of Biology "Consiglio Nazionale delle Ricerche".

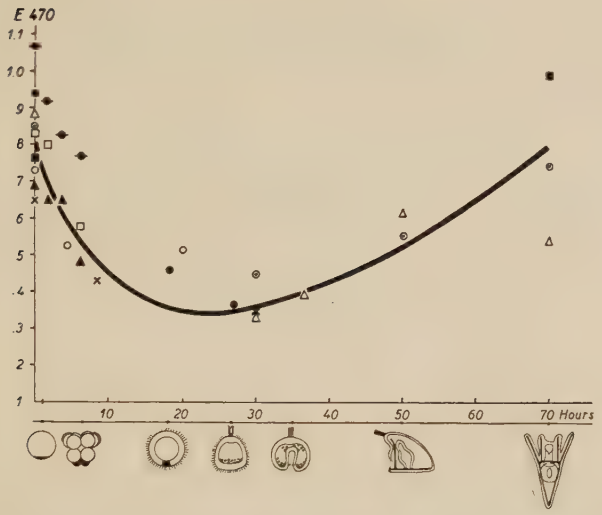

Fig. 1. Changes in the total content of carotenoids during development of *Paracentrotus lividus*. E_{max} of the extract of the pigment is referred to 10 mg N. Each symbol refers to a single experiment.

Spectrophotometer between 400 and 500 mμ. The maxima of the spectra were compared and for the final calculation the highest absorption point, i.e. between 470 and 475 mμ, was taken as a measure of the quantity of pigment present in the extract.

From each one batch of eggs used for the extraction of the pigment, two samples of 0.2 ml for N determination and 4 samples for the egg count (using a Linderstrøm-Lang-Holter calibrated mouth pipette) were taken. The reference points for the quantity of pigment in the extracts were both total N and number of eggs.

RESULTS

Fig. 1 shows that a conspicuous decrease of the total quantity of pigment takes place between fertilization and the early gastrula stage. From the gastrula stage on, the quantity of pigment is found to increase again. Boveri (1) had already found that the cells of the primary mesenchyme are pigment-free. Since the micromeres, which are the source for the primary mesenchyme, often are pigmented until the moment preceding their invagination, the pigment probably disappears during invagination. In the late gastrula stage, amoeboid cells, rather heavily loaded with large pigment granules of a brick-red colour, make their appearance. These pigment granules differ from those of the egg both with respect to their larger size and to their colour. The number of these cells rapidly increases with the age of the embryo. Some of them seem to move freely in the body cavity. Some others are scattered among or just below the cells of the ectoblast and of the wall of the primary intestine. These cells were also described by Boveri (1) who considered the pigment as being

of the same nature as the pigment of the egg. We have found that starting from the stage when the new pigment makes its first appearance, the embryos still retain a red-violet colour after having been exhaustively extracted in the aforementioned way. At the microscope, one can see that the colour is due to the pigment still present in the body of the amoeboid cells. The pigment cannot be extracted with chloroform, acetone, methanol, pyridine, but on slight acidification with dilute HCl, it can be taken up quantitatively in ether. The absorption spectrum in ether exhibits maxima at about 415, 455, 565 mμ. However, owing to the low concentration of the pigment available, the spectrum needs to be checked again. We can add that the pigment turns to red in acid solution and to violet in the alkaline range. Probably this is the echinochrome (or rather a precursor of it) that, as known, in the adult *Paracentrotus* is present in the cells of the perivisceral fluid.

In one case the eggs of a female developed apparently normally up to the beginning of gastrulation, when they exogastrulated. In this case practically no change of the total quantity of pigment was found during development nor was "echinochrome" formed.

The results reported above suggest that the metabolism of the carotenoid pigments in the eggs of *Paracentrotus lividus* may constitute an important step in the early morphogenesis, especially in the processes underlying gastrulation. They do not give, however, any indication as to the pathway along which the pigment is metabolized. Experiments to investigate this question are under way.

SUMMARY

In the eggs of *Paracentrotus lividus* the total quantity of pigment appears to decrease during the early stages of development, i.e. between fertilization and gastrulation. Data are also given concerning the appearance of a new pigment, possibly a precursor of the echinochrome.

REFERENCES

1. Boveri, Th., *Zool. Jahrb. Abt. Anat. Ontog.*, **14**, 630 (1901).
2. Karrer, P., and Jucker, E., Carotinoide. Birkhäuser Verlag, Basel, 1948.
3. Monroy, A., and Runnström, J., *Biol. Bull.*, **99**, 339 (1950).

SAKAGUCHI OXINE TEST FOR ARGININE APPLIED TO TISSUE SECTIONS[1]

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Sakaguchi introduced the original biochemical test for arginine in 1925 (8). Adaptations for histochemical use were made by Baker (1), Serra (10) and Thomas (11). These methods used a-naphthol as the reacting substance. After proper preparation of mammalian tissues the development of an orange or orange-red color indicates the presence of arginine alone (2, 12). The color produced is admittedly unstable. The procedures are complicated.

Recently Sakaguchi described a second test for arginine (9). 8-hydroxyquinoline (oxine) was used in place of a-naphthol. The present paper describes a method using oxine in histochemical demonstration of arginine. This simple procedure produces a relatively stable orange color. The method lends itself to quick screening of tissues in a survey for the presence of arginine. Results of preliminary studies on a number of tissues are included.

REAGENTS

1. 8-hydroxyquinoline (oxine), made to 0.4 per cent in absolute alcohol.
2. 10 per cent sodium hydroxide.
3. Sodium hypochlorite.

METHODS

Tissues to be examined are fixed for 24 hours in 10 per cent formalin or in an alcohol-formalin mixture prepared as follows (7):

95 per cent ethyl alcohol 90 cc, 37 per cent formaldehyd 10 cc, 0.1 Normal sodium hydroxide 1 cc. Neither Bouin's nor Zenker's fixatives were used.

[1] Aided by a grant from National Institute of Health. This paper was read in part before the Histochemical Society, Detroit, March, 1951.

[2] Surgeon, U.S. Public Health Service. Member of Pathology Section, National Cancer Institute, National Institute of Health, Bethesda, Maryland.

After thorough washing in water, the tissue block is cut on a freezing microtome. Fairly thick sections (15–20 microns) give better coloring.

The oxine reaction mixture is prepared in a small container in the following pro portions:

3 drops 0.4 per cent oxine solution, 20 drops distilled water, 20 drops 10 per cent sodium hydroxide, 5 drops sodium hypochlorite.

For thymus gland and other delicate tissues the mixture is prepared with half the usual amount of sodium hypochlorite. The reaction mixture deteriorates rapidly. It must be remade if not used within twenty minutes. A tissue section is placed in the reaction mixture and the color allowed to develop with gentle agitation. Strongly positive tissues show a deep orange color in one minute. Other tissues are slower to produce the color. An arbitrary period of two minutes is selected as the reaction time. Longer exposure results in some tissue decomposition.

The tissue is then very quickly taken through alcohols for dehydration, briefly drained on a filter paper and immersed in a glycerine bath. Here the tissue can be easily unfolded. It is mounted on a slide, using the glycerine as a mounting medium.

An alternate procedure giving good results is the preparation of mounted paraffin sections from alcohol-formalin fixed tissues. The paraffin is not removed (11). The slide is placed in a horizontal position and flooded with the reaction mixture. Renew the mixture if necessary. Development of the color very nearly parallels that of the previous method. The same period of two minutes is used. After passing the section through dehydrating alcohols rapidly the tissue is mounted in glycerine.

The orange color developed by either of these methods is relatively stable. Some sections have been observed to retain a good color for three or four days.

RESULTS

Various tissues from humans, guinea pigs, rats, and rabbits were examined in a preliminary survey. The results are given in Table I. In general, the best orange color was developed in connective tissues and in tissues known to have a high arginine content. Epithelium and epithelial tumors have a low arginine content.

Some glandular structures are surprisingly rich in arginine. Duodenal mucosa develops a moderately heavy color. Thyroid colloid usually has a good color. The deepest orange color observed was in thymus gland.

Fibrous connective tissue and smooth muscle give bright colors wherever encountered (Figure 1), as in vessel walls or in uterine leiomyomata. Capsules of some organs, as liver, kidney, and spleen, are much brighter orange than the parenchyma. Subcutaneous tissue, rich in collagen, contains much more arginine than the overlying epidermis (Figure 2).

DISCUSSION

The colors were found to be consistent and reproducible under various conditions. The execution of this reaction at ice bath temperatures as recommended by Thomas (12) was not considered essential for this simplified method. Under certain circumstances the development of the arginine color at ice bath temperatures might be

TABLE I

Results: Oxine—Arginine Histochemical Method.

Tissue	Human	Guinea Pig	Rat	Rabbit
Collagen	+	+	+	+
Smooth Muscle	+	+	+	+
Thyroid colloid	+	— —	— -	— —
Brunner's glands	+	+	— —	— -
G. I. Mucosa	+	+	— —	— —
Wharton's Jelly	+	— —	— —	— —
Nevus Cells	+	— —	— -	— —
Myelin sheaths	+	— -	— -	— —
Thymus Follicles	+	— —	— —	+
Spleen, Germinal Centers	+	+	— —	— -
Epidermis	+	— -	— —	— —
Adrenal	0	— —	— -	— —
Prostate cells	0	— —	— —	— —
Thyroid cells	0	— —	— —	— —
Blood	0	0	0	0
Spleen	0	0	— —	0
Liver (Parenchyma)	0	0	0	0
Kidney (Parenchyma)	0	— —	+	0
Lung (Parenchyma)	0	0	+	— —
Pancreas (Parenchyma)	0	— —	— —	— —
Cartilage Matrix, Ear	— -	— —	— —	+
Several Epithelial Tumors	0	— —	— —	— -
Testis	+	— -	— —	— —

+ = positive.
0 = negative.
— — — not done.

recommended. Urea was not used to halt the action of hypochlorite. The preparation of the reaction mixture in one container and the rapid transfer of the tissues through alcohol to glycerine rendered this step unnecessary. The orange color can easily be lost in the dehydrating alcohols. For this reason the immersion time of the tissue in alcohol after production of color must be very brief. The routine procedure described is a simple technique. It gives excellent results for non-permanent histochemical demonstrations.

The advantages of keeping the tissue in paraffin in the alternate procedure are the maintenance of the shape of the section and the preservation of cellular detail. The tissue clears fairly well in the glycerine mounts. The paraffin usually presents no obstacle for studying the prepared slide. Occasionally the paraffin section may contain many air bubbles or may be so dry and brittle that cracks appear. These

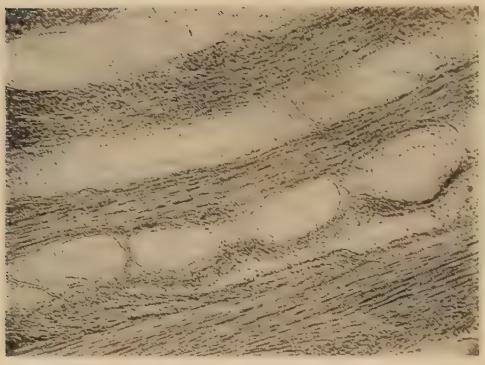

Fig. 1.

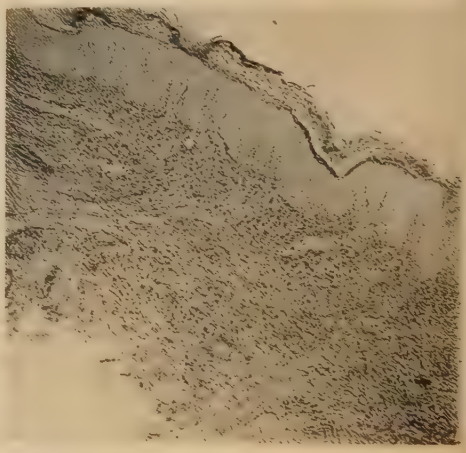

Fig. 2.

Fig. 1. Human Prostate, formalin fixation, frozen section. Depth of orange color is suggested by the darker appearing connective tissue stroma. The glandular cells are negative (Wratten B filter No. 58; 120 ×).

Fig. 2. Human Skin, formalin fixation, frozen section. The epidermis is lightly stained, while collagen-rich subcutaneous tissues have a deeper color. The keratin layer is the most positive of the epidermis (Wratten B, filter No. 58; 75 ×).

defects interfere with the study of the finished slide. It has been our experience, however, that added manoeuvres to dissolve the paraffin cause destruction of the tissues on the slide.

Several other modifications of the procedure have been tried with varying degrees of success. A more permanent section can be prepared by using the new Technicon[1] mounting fluid after more careful dehydration. Brun's glucose mounting medium turned the arginine color brown. Glycerine jelly has no advantage over glycerine as a mounting medium.

Several interesting slides were prepared with tissues fixed in 2 per cent osmium tetroxide (6). Cellular detail was enhanced.

Results of the preliminary investigation of some tissues are in keeping with the findings expected. The deepest color obtained was in thymus gland sections. Hamer found that 30.7 per cent of the total protein nitrogen in calf thymus histone was due to arginine (5). Duodenal mucosa appears to be more rich in arginine than other sections of the gastrointestinal tract or than other epithelial tissues. Granick found high concentrations of apoferritin in this site (4). Block and Bolling give the arginine content of ferritin as 17.7 per cent (3). Colloid in the thyroid gland is moderately heavily colored. The arginine content of thyroglobulin is 7.4 per cent (3). The strong colors produced in connective tissue prove to be very interesting, as did the faint colors in epithelial tissues and the carcinomas studied.

[1] Courtesy The Technicon Company, New York.

SUMMARY

A simplified histochemical method of demonstrating the presence of arginine in tissues is described. This procedure is modified from Sakaguchi's second test for arginine, using 8-hydroxyquinoline (oxine).

Good orange colors in tissue sections, denoting the presence of arginine, were found in connective tissues, thymus gland, duodenal mucosa, and the thyroid colloid. Epithelial tissues and epithelial tumors generally contained little arginine by this method.

ACKNOWLEDGEMENT

The authors wish to express appreciation to Mrs. Ethel V. Hubbard for her excellent technical assistance.

REFERENCES

1. Baker, J. R., *Quart. J. Microscop. Sci.*, **85**, 1 (1944).
2. —— *Quart. J. Microscop. Sci.*, **88**, 115 (1947).
3. Block, R. J., and Bolling, D., The Amino Acid Composition of Proteins and Foods. Charles C. Thomas, Springfield, Illinois, 1945.
4. Granick, S., *J. Biol. Chem.*, **164**, 737 (1946).
5. Hamer, D., *Nature*, **167**, 40 (1951).
6. McManus, J. F. A., and Lupton, C. H., Jr., Unpublished results.
7. Ritter, H. B., and Oleson, J. J., *Arch. Path.*, **43**, 330 (1947).
8. Sakaguchi, S., *J. Biochem. (Japan)*, **5**, 25 (1925).
9. —— *ibid.*, **37**, 231 (1950).
10. Serra, J. A., *Naturwissenschaften*, **32**, 46 (1944).
11. Thomas, L. E., *J. Cellular Comp. Physiol.*, **28**, 145 (1946).
12. —— *Stain Technol.*, **25**, 143 (1950).

CELL-WALL STRUCTURE IN *CHLAMYDOMONAS*[1]

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ELECTRON microscope studies of the fine structure of cellulose have been handicapped by the opacity of most plant cell-walls to the electron beam. This difficulty has been overcome to some extent by the use of cellulose material, such as plant fibres, which has been subjected to somewhat drastic processes of mechanical or chemical disintegration (3), or by the study of the cellulose pellicle formed by *Acetobacter xylinum* (4). However, preparations of comparatively undamaged cell-walls (e.g. of *Zea* root meristems) have been examined and illustrated by Frey-Wyssling *et al.* (1).

The cell-walls of green algae are stated generally to be composed of cellulose (6). Those of *Valonia ventricosa* (Siphonales) provide a favourable subject for investigation, since the coenocytes are large and easily handled. Electron microscope studies of the fine structure have been carried out, using surface replicas (5), or direct mounts of lamellae separated by microdissection of macerated cell-walls (8).

In many unicellular algae, at each cell division, individual walls are formed around the daughter cells; and when the young cells are liberated, the empty parental wall remains. Such shed walls provide suitable material for direct examination under the electron microscope.

Chlamydomonas moewusii (Volvocales) grows well in mineral media under suitable conditions of illumination (2). In every culture there is abundant cell-wall material in various stages of natural disintegration. Whole cells can be removed from such a culture by a brief period (3 min.) of centrifugation at 350 g: a longer period (20 min.) spins down the cell-walls, which may be washed by repeated centrifugation in distilled water. The walls shown were mounted by drying on collodion film supported by a wire screen, and shadowed with palladium at an angle of 7 to 1.

The cell-walls of this alga are extremely thin and transparent to the electron beam. Examination of such walls under the electron microscope has revealed a striation of the surface, the fundamental period being of the order of 233 $\pm$ 6 Å. In several different preparations the total width of a clearly visible series of striations (usually 20–50) was measured by the use of a calibrated ocular micrometer and from this measurement the average band width was calculated. If this indicates a regular parallel orientation of cellulose fibrils, it is noteworthy that they are somewhat narrower than those hitherto observed in cellulose preparations [e.g. 300 Å. (5); 250–400 Å. (3)].

[1] Aided by a grant from the Fluid Research Fund, Yale University School of Medicine.
[2] Aided by a Theresa Seessel post-doctoral fellowship.

Fig. 1. Empty cell-wall of *Chlamydomonas moewusii*, collapsed and somewhat folded. The flagellar pores and two short portions of flagella are indicated by arrows. ×10 500 (E. M. 2240 A).

Similar striation of the cell-wall is exhibited by *Chlamydomonas dysosmos*. No striation has been observed in *Selenastrum gracile* (Chlorococcales), in agreement with observations of Sponsler and Bath (7) on the related alga *Ankistrodesmus falcatus*.

(Cultures of algae are gratefuliy acknowledged from the following:

Chlamydomonas ? *dysosmos* Moewus ... Mr. I. C. G. Cooper
Chlamydomonas moewusii Gerloff ..... Dr. L. Provasoli
Selenastrum gracile Reinsch ..... Miss Naomi Cowen.)

SUMMARY

Cell-walls of *Chlamydomonas moewusii* exhibit under electron microscopy a regular striation with a period of 233 Å. It is presumed that this indicates parallel orientation of cellulose fibrils.

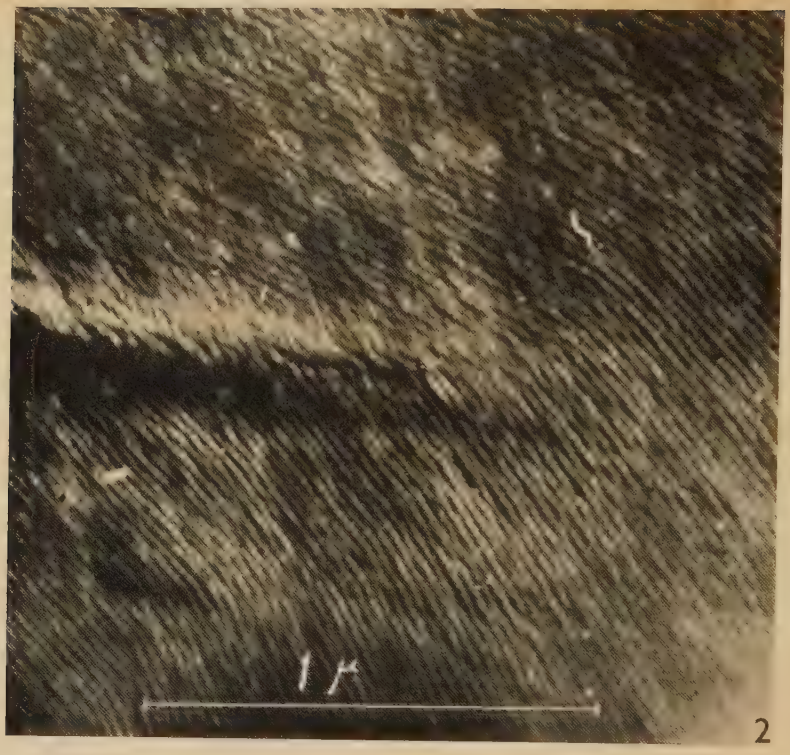

Fig. 2. Portion of wall. × 60 000 (E. M. 2467 E). (Surface replica of cell-wall.)

REFERENCES

1. Frey-Wyssling, A., Mühlethaler, K., and Wyckoff, R. W. G., *Experientia*, **4**, 475 (1948).
2. Lewin, R. A. (in press).
3. Mühlethaler, K., *Biochim. et Biophys. Acta*, **3**, 15 (1949).
4. —— *Biochim. et Biophys. Acta*, **3**, 527 (1949).
5. Preston, R. D., Nicolai, E., Reed, R., and Millard, A., *Nature*, **162**, 665 (1948).
6. Smith, G. M., Freshwater Algae of the United States. 2nd ed., McGraw-Hill, New York, 1950.
7. Sponsler, O. L., and Bath, J. D., *Amer. J. Bot.*, **36**, 756 (1950).
8. Wilson, K., *Ann. Bot.*, **15**, 279 (1951).

ABSORPTION OF X-RAYS IN INHOMOGENEOUS HISTO- AND CYTOLOGICAL SAMPLES

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Absorption measurements with X-rays have proved to be of value in histo- and cytochemical analysis. A factor which may complicate the quantitative interpretation of absorption data obtained with any kind of electromagnetic radiation is the inhomogeneous distribution of absorbing substance. This is very often the case in histo- and cytochemical analysis of biological material. The effect of the inhomogeneity of a sample on the accuracy of the absorption measurements with X-rays is dealt with in the present communication.

Let the medium have a linear absorption coefficient μ (cm^{-1}), density ϱ ($g\ cm^{-3}$) and surface density q ($g\ cm^{-2}$). The linear absorption coefficient is composed of two terms, $\mu = \tau + \sigma$, where τ refers to photoelectric absorption and σ to scattering. For X-rays of the wavelength 12 Å, τ has the value 2000 for a soft tissue but σ only about 0.1 per cent of that value. Therefore in the region most commonly used for histo- and cytochemical analysis by X-rays, 5 to 50 Å, effects of scattering may be neglected in comparison with photoelectric absorption. The attenuation of a parallel beam of X-rays of initial intensity I_0 entering a homogeneous medium at normal incidence is expressed by:

$$I = I_0 \cdot e^{-\frac{\mu}{\varrho} \cdot q} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad 1$$

However the same total amount of absorbing substance can be present in the same cross sectional area but inhomogeneously distributed. For the further calculations it is assumed that the sample is broken up into absorbing and nonabsorbing regions. It is assumed that the aperture of the detector is always larger than the diameter of the beam which in turn is larger than the mean diameter of any of the absorbing regions. It is further assumed, for purpose of simplification, that the absorbing regions are of spherical shape and not overlapping. Consider first the absorption of X-rays in a sphere. Let the radius of the sphere be R. The average intensity of X-rays after passing through the sphere is:

$$I_{AV} = I_0 \cdot \frac{1}{\pi R^2} \int_0^R e^{-2\mu\sqrt{R^2 - r^2}} \cdot 2\pi r\, dr \qquad \ldots\ldots\ldots\ldots\ldots \quad 2$$

[1] Rockefeller Fellow.

After integration we obtain:

$$I_{AV} = I_0 \cdot \frac{1}{2\mu^2 \cdot R^2}\left[1 - e^{-2\mu R}(2\mu R + 1)\right] \quad \ldots\ldots\ldots\ldots 3$$

The original, homogeneous sample is assumed to have the shape of a parallelepiped with the volume V (cm^3), cross sectional area A (cm^2), density ϱ ($g\,cm^{-3}$) and surface density q ($g\,cm^{-2}$). The absorbing substance within that volume is concentrated into m small spheres with the radius R. If the linear absorption coefficient is μ for the original sample the linear absorption coefficient μ_s for the spheres is:

$$\mu_s = \mu \cdot \frac{V}{m \cdot \frac{4}{3} \cdot \pi \cdot R^3} = \frac{\mu}{\varrho} \cdot q \cdot \frac{3}{4} \cdot \frac{A}{\pi \cdot R^3 \cdot m} \quad \ldots\ldots\ldots\ldots 4$$

If $\frac{A}{\pi R^2} = p$, we get:

$$\mu_s = \frac{\mu}{\varrho} \cdot q \cdot \frac{3p}{4 \cdot R \cdot m} \quad \ldots\ldots\ldots\ldots 4\,a$$

It is clear that of the area A of the homogeneously absorbing material, now $m\frac{\pi R^2}{A}$ is absorbing and $\left[1 - m\frac{\pi R^2}{A}\right]$ nonabsorbing. Thus we can write that one fraction of the beam, I_1, has not been absorbed,

$$I_1 = I_0\left(1 - \frac{m}{p}\right) \quad \ldots\ldots\ldots\ldots 5$$

and the other fraction, I_2, is absorbed by the spheres,

$$\bar{I}_2 = I_0 \frac{m}{p \cdot 2 \cdot \mu_s^2 \cdot R^2}\left[1 - e^{-2\mu_s R}(2 \cdot \mu_s \cdot R + 1)\right] \quad \ldots\ldots\ldots\ldots 6$$

The sum of the last two expressions is the intensity of the radiation transmitted through an inhomogeneous sample. After inserting the value for μ_s we get:

$$\bar{I} = I_1 + I_2; \frac{\bar{I}}{I_0} = \frac{8 \cdot m^3}{9 \cdot p^3 \cdot \left(\frac{\mu}{\varrho} \cdot q\right)^2}\left[1 - e^{-\frac{3 \cdot \mu \cdot p \cdot q}{2 \cdot \varrho \cdot m}}\left(\frac{3 \cdot \mu \cdot p \cdot q}{2 \cdot \varrho \cdot m} + 1\right)\right] + 1 - \frac{m}{p} \quad \ldots\ldots 7$$

In equation 7 the value of $\frac{\bar{I}}{I_0}$ is measured experimentally. In order to obtain $\left(\frac{\mu}{\varrho} \cdot q\right)$, $\frac{m}{p}$ has to be estimated. The value of $\frac{m}{p}$ can be determined from a high power photomicrograph of the area under analysis. The total number of absorbing structures, which can be counted is m, and the ratio of one of these areas to the beam area is $\frac{1}{p}$.

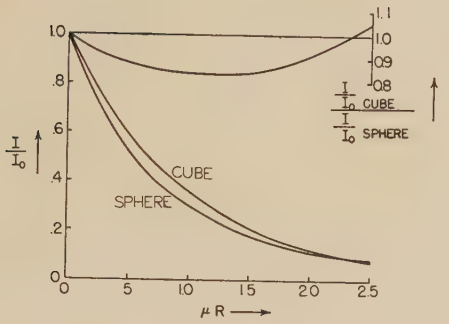

Fig. 1.

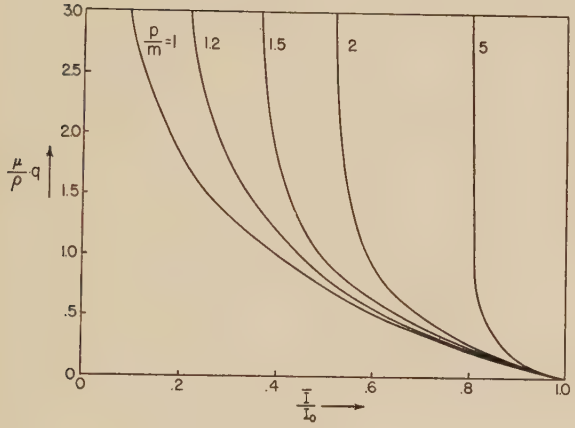

Fig. 2.

It is then assumed that all small particles have the same size and are non-overlapping spheres. If the particles have different size Eq. 7 can be applied to groups of particles having the same size. In general not too great errors will occur if $\frac{1}{p}$ is taken to be the mean area of the particles in ratio to the beam area. The shape of the particles may deviate from that of a sphere. If it is assumed that the sample is concentrated into small cubes of side $2\,R$ instead of spheres of radius R and with the same total amount of absorbing substance the linear absorption coefficient for the cubes is $\frac{\pi}{6}$ times that for the spheres. The absorption of a sphere and a cube with the diameter and side length $2\,R$ respectively and containing the same amount of absorbing substance is shown in Fig. 1. The X-ray beam has the same cross sectional area as the sphere and cube respectively. It can be seen that the errors in transmission are not too great if the particles in the sample deviate somewhat from the spherical shape. In a biological sample the particles mostly have nearly spherical shape.

In Fig. 2, $\left(\frac{\mu}{\varrho} \cdot q\right)$, is plotted against $\frac{\bar{I}}{I_0}$ for different values of $\frac{p}{m}$. If the measured value of $\frac{I}{I_0}$ is 0.5 and $\frac{p}{m}$ is 1.5 the curve gives $\left(\frac{\mu}{\varrho} \cdot q\right) = 0.94$. If no attention had been paid to the inhomogeneous distribution a value of $\left(\frac{\mu}{\varrho} \cdot q\right) = 0.69$, which corresponds to $e^{-\frac{\mu}{\varrho} \cdot q} = 0.5$ would have been obtained thus giving a large error.

The calculation is performed under the assumption that parallel X-rays are used for the absorption measurement. When the microradiographic technique is used for imaging a biological sample the X-rays have a certain divergence. In a typical case, however, the divergence does not exceed 5°, in general it is lower. Thus Eq. 7 can still be used with good accuracy. In the X-ray microscope the divergence of the X-rays is in the magnitude 0.5° and Eq. 7 is valid in this case too.

SUMMARY

A formula has been derived showing the effect of inhomogeneously distributed absorbing substance on the accuracy of X-ray microabsorption measurements.

These studies were carried out in part under a grant from the Alfred P. Sloan Foundation.

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